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# **Microbial Colonisation and Degradation of Plastic Pollution in the Marine Environment**

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B.Sc. (Hons), M.Phil.

A thesis submitted for the degree of Doctor of Philosophy

September 2019

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## **Author's Declaration**

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# Abstract

Plastic pollution is ubiquitous in the world's oceans and is predicted to increase by an order of magnitude by 2025. The environmental impacts of plastic pollution are well documented in terms of its effects on marine animals however, the impact plastic has on the natural physicochemical characteristics of seawater and the microbial composition of the world's oceans remains largely unknown. In particular, the role that microbial communities play in the early stages of colonisation and decomposition requires more attention. In response to this knowledge gap, both laboratory-based and field work experiments were undertaken in order to gain further insights. A microcosm experiment using natural seawater amended with range of plastics, both synthetic and biodegradable, was carried out to evaluate the impact of these plastics on the marine microbial communities. To complement this, a one-year field exposure trial was undertaken to measure biofilm formation and growth, as well as changes in polymer characteristics resulting from degradation. Finally, a biodegradation study was undertaken to identify bacteria within seawater that may degrade plastic.

The characterisation of marine bacterial communities revealed major shifts in composition when plastics were introduced into seawater. These microbiological shifts were due to changes in the concentration of dissolved organic carbon, pH and total nitrogen due to the degradation of plastic materials. Furthermore, the long-term field work exposure experiment showed that different plastic substrates selected for distinct colonising communities on their surfaces. The analysis of the biofilm communities revealed the presence of bacteria that could possibly be involved in the degradation of plastics. However, no significant degradation was measured for the *in situ* tested plastics, suggesting that biofilm formation may have limited degradation. An exploration of plastic degradation using distinct bacterial consortia isolated from seawater resulted in biodegradation (mineralisation) figures of 16, 9 and 7 % for polyvinyl chloride, high-density polyethylene and polyethylene terephthalate respectively.

In summary, results from this thesis suggest that the presence of plastic in seawater affects the microbial community by changing the inherent chemical and biological properties of the water. In addition, plastics were shown to select for distinct colonising communities, which did not contribute to the plastic degradation. However, enriched marine bacterial communities demonstrated biodegradation ability that could be explored further in the future to gain additional insights into underlying biodegradation mechanisms.

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# Contents

<b>Author's Declaration .....</b>	<b>i</b>
<b>Abstract.....</b>	<b>ii</b>
<b>Acknowledgements.....</b>	<b>iii</b>
<b>Contents .....</b>	<b>iv</b>
<b>List of Figures.....</b>	<b>vii</b>
<b>List of Tables .....</b>	<b>xi</b>
<b>List of Abbreviations .....</b>	<b>xiii</b>
<b>Chapter 1: Introduction .....</b>	<b>1</b>
1.1 Importance of the Research Area .....	1
1.1.1 Plastic Entanglement and Ingestion.....	1
1.1.2 Plastic Toxicity .....	2
1.1.3 Economic Effects.....	3
1.2 Objectives.....	4
1.3 Literature Review .....	6
1.3.1 Production.....	6
1.3.2 Origin and Levels of Plastic Pollution in the Marine Environment .....	8
1.3.3 Degradation of Plastics in the Marine Environment.....	10
1.3.3.1 Photodegradation .....	11
1.3.3.2 Thermal degradation .....	13
1.3.3.3 Hydrolysis.....	15
1.3.3.4 Mechanical Degradation .....	17
1.3.3.5 Biodegradation.....	17
1.4 Interactions Between Marine Microorganisms and Plastics .....	19
1.4.1 Potential Impacts of Plastics on Marine Microorganisms .....	19
1.4.2 Microbial Attachment and Biofilm Formation on Marine Plastics .....	21
1.4.3 Plastic Degradation by Marine Microbes .....	23
<b>Chapter 2: A Time-Series Investigation into the Effects of Plastic Pollution on Microbial Community Structure and Diversity in Seawater: A Microcosm Study</b>	<b>27</b>
2.1 Introduction .....	27
2.2 Materials and Methods .....	29
2.2.1 Seawater Sampling .....	29
2.2.2 Seawater-Plastics Microcosms Setup .....	30
2.2.3 Physico-Chemical Characterisation of Seawater.....	31
2.2.3.1 Seawater pH Measurements .....	31
2.2.3.2 Dissolved Inorganic Carbon (DIC), Dissolved Organic Carbon (DOC) and Total Nitrogen (TN) Determination.....	31
2.2.4 Microbial Community Analysis .....	32
2.2.4.1 Seawater Cell Enumerations.....	32
2.2.4.2 Terminal Restriction Fragment Length Polymorphism (T-RFLP).....	32
2.2.4.3 Genomic DNA Extraction .....	33
2.2.4.4 PCR Amplification and Product Digestion.....	33
2.2.4.5 TRFs Data Processing.....	34
2.2.4.6 Illumina MiSeq Barcoded Amplicon Sequencing .....	34
2.2.4.7 <i>In Silico</i> Digestion of MiSeq Sequences .....	35
2.2.5 Statistical Analysis.....	35
2.3 Results .....	37
2.3.1 Physico-Chemical Characterisation of Seawater.....	37
2.3.1.1 Seawater pH.....	37
2.3.1.2 DOC, DIC and TN Determination.....	37

2.3.2 Microbial Community Analysis.....	39
2.3.2.1 Seawater Cell Enumerations .....	39
2.3.2.2 Microbial Diversity and Community Structuring based on T-RFLP Profiles .....	40
2.3.2.3 Substrate Specificity of the Seawater Microbiome .....	42
2.3.2.4 Relationship of the microbial communities with environmental variables .....	50
2.4 Discussion.....	52
2.5 Conclusions.....	58
<b>Chapter 3: Diversity and Dynamics of Microbial Community Succession on Synthetic Plastics in Seawater: A long-term Marine Exposure Study .....</b>	<b>60</b>
3.1 Introduction.....	60
3.2 Materials and Methods.....	62
3.2.1 Experimental Setup and Sampling .....	62
3.2.2 Biofilm Assays .....	63
3.2.3 Scanning Electron Microscopy .....	64
3.2.4 DNA Extraction and Next-Generation Sequencing .....	64
3.2.5 MiSeq Data Processing .....	65
3.2.6 Statistical Analysis .....	66
3.3 Results.....	67
3.3.1 Biofilm Formation and Quantitation .....	67
3.3.2 Scanning Electron Microscopy of Microbial Biofilm on Plastics .....	70
3.3.3 Dynamics of Bacterial Community Composition .....	75
3.3.4 Structuring and Specificity of Bacterial Communities .....	76
3.3.5 The Core Biofilm Communities.....	78
3.4 Discussion.....	81
3.5 Conclusions.....	85
<b>Chapter 4: Degradation of synthetic plastics in the sea: a long term <i>in situ</i> exposure study.....</b>	<b>86</b>
4.1 Introduction.....	86
4.2 Materials and methods .....	88
4.2.1 Experimental Design.....	88
4.2.2 Removal of Biofilm .....	88
4.2.3 Measuring Plastic Degradation .....	89
4.2.3.1 Plastic Surface Characterisation.....	89
4.2.3.1.1 Scanning Electron Microscopy .....	89
4.2.3.1.2 Surface Wettability Analysis.....	89
4.2.3.2 Mechanical Characterisation .....	89
4.2.3.2.1 Tensile Stress Testing .....	89
4.2.3.3 Spectroscopic Analysis .....	91
4.2.3.3.1 Fourier Transform Infrared (FTIR) Spectroscopy .....	91
4.2.4 Statistics .....	92
4.3 Results.....	92
4.3.1 Temperature and Light.....	92
4.3.2 Morphological Changes .....	93
4.3.3 Contact Angle Measurement.....	98
4.3.4 Changes in Tensile Properties.....	99
4.3.5 Changes in ATR-FTIR Spectra.....	101
4.4 Discussion.....	106
4.5 Conclusions.....	110
<b>Chapter 5: Biodegradation of Synthetic Plastics by Marine Consortia: A Respirometric Study.....</b>	<b>111</b>
5.1 Introduction.....	111

5.2 Materials and methods.....	113
5.2.1 Seawater Sampling .....	113
5.2.2 Enrichment of Marine Consortia .....	113
5.2.3 Selection and Isolation of Marine Bacteria with the Plastic Degrading Ability .....	114
5.2.4 Molecular Identification of Isolates.....	114
5.2.5 Biodegradation Test.....	115
5.2.5.1 Respirometric System .....	116
5.2.5.2 Preparation of Inoculum .....	117
5.2.5.3 Preparation and Inoculation of Respirometry Vessels.....	118
5.2.5.4 Calculations .....	118
5.2.6 Analysis of Physicochemical Properties.....	119
5.3 Results .....	120
5.3.1 Preliminary Screening of Plastic Degrading Marine Bacterial Isolates .....	120
5.3.2 Identification of Isolates .....	121
5.3.3 Respirometric Analysis.....	122
5.3.4 Surface Morphology of the Plastics.....	124
5.3.5 Changes in ATR-FTIR Spectra .....	126
5.4 Discussion .....	129
5.5 Conclusions .....	133
<b>Chapter 6: General Discussion .....</b>	<b>134</b>
6.1 Effect of Plastics on Marine Microorganisms .....	134
6.2 Marine Biofilm Formation on Plastics .....	137
6.3 Physical Degradation of Plastics .....	139
6.4 Biodegradation of Plastics.....	140
6.5 Recommendations for future work.....	142
6.6 Conclusions .....	143
<b>Appendices .....</b>	<b>145</b>
Appendix A. SIMPER analysis of bacterial communities.....	145
Appendix B. PERMANOVA pair-wise tests.....	146
Appendix C. PERMDISP pair-wise tests .....	147
Appendix D. Rarefaction curves of seawater bacterial communities.....	148
Appendix E. Rarefaction curves of HDPE biofilm communities.....	148
Appendix F. Rarefaction curves of PET biofilm communities .....	149
Appendix G. Rarefaction curves of PVC biofilm communities .....	149
<b>References .....</b>	<b>150</b>

# List of Figures

Figure 1.3.1 Chemical structures of some common plastics and their functional groups .....	7
Figure 1.3.2 Trends of global plastics production. (a) Worldwide and European plastics production in million metric tons per year (Mt year <sup>-1</sup> ) from 1950 to 2017. (b) Plastic applications patterns in percent during 2017. Data compiled from Geyer et al. (2017) and Plastics Europe (2018). .....	8
Figure 1.3.3 The pathways of plastic waste into the marine environment. The direct sources of plastic waste from sea-based activities are shown in green shading, from land-based activities in purple, and from either maritime or land activities in orange. Redrawn and adapted from (Hammer et al., 2012, Law, 2017). .....	9
Figure 1.3.4 Random Scission mechanism for thermal degradation of plastics .....	14
Figure 1.3.5 The hydrolytic cleavage of an ester bond under (a) Acidic and (b) Alkaline conditions. ....	16
Figure 1.3.6 Various steps of plastic biodegradation in the marine environment. Original diagram by (Wei and Zimmermann, 2017).....	19
Figure 1.4.1 Overview of the potential impact of plastic on the regulation of carbon and other nutrient cycles. Dissolve (DOM) and particulate (POM) organic matter originated from the linear trophic chain is returned to higher trophic levels via its incorporation in bacterial biomass. Original diagram by Jacquin et al. (2019).....	20
Figure 1.4.2 Stages of biofilm formation and development. Redrawn and adapted from (Flemming, 1998, Rendueles and Ghigo, 2012) .....	23
Figure 2.2.1 Location of the seawater sampling site. The small black box denotes the regional location of the sampling sites within the UK. Location of field site used for seawater sampling at Skegness, Lincolnshire, UK is shown in the magnified box. Circular marker denotes the sampling site. ....	29
Figure 2.3.1 Profiles of physico-chemical parameters of NSW during plastic treatments: (a) pH, (b) DOC, (c) DIC and (d) TN. Each parameter plotted over sampling time for three replicates. Error bars represent the standard deviation for the average measurement at each sampling point.....	38
Figure 2.3.2 Bacterial cell numbers during plastic treatments. Bacterial cells (mL <sup>-1</sup> ) plotted over sampling time for three replicates in seawater-plastic treatments. Error bars represent the standard deviation for the average cell count at each sampling point.....	40
Figure 2.3.3 The Shannon-Weiner diversity indices are presented here as a boxplot comparing the $\alpha$ -diversity between NSW and plastic treatments (a) and time points (b) measured in this study.....	40
Figure 2.3.4 Principle Coordinate Ordinations (PCO) indicating variation in the bacterial community composition at ten sampling points. PCOs representing the similarity of the bacterial communities across all plastic treatments and the control, based on Bray-Curtis resemblance matrix. Circles indicate the percentage of similarity between samples based on cluster analysis and labels correspond to sampling time points in hours.....	42

Figure 2.3.5 Bacterial community composition at the family-level classification of NSW sample collected for the study. Only families that represented at least 1% of the community are shown. ....	44
Figure 2.3.6 Bacterial community composition of NSW at family-level classification for each of the treatments over the 512 hrs experiment run. Only families representing at least 1% of the community are shown. ....	48
Figure 2.3.7 Distance-based redundancy analysis (dbRDA) plot to visualise DistLM results in 2-dimensional space for individual environmental variables. Vectors length is proportional to their contribution to the total variation and the circle denotes a correlation of 1.0. ....	52
Figure 3.2.1 Map of the exposure study site. The small black box denotes the regional location of the site within the UK. Location of field site at Plymouth, Devon, UK is shown in the magnified box. Grey colour represents land and white represents the sea. Circular marker denotes the sample deployment site. ....	62
Figure 3.2.2 The deployment structure used to expose plastics in natural marine water. (a) the plastic films holding frame, (b) three strips of each plastic type secured between the nylon micromesh within each holding frame, (c) the arrangement of the sample holding frames mounted on 15 mm thick PVC supporting beam.....	63
Figure 3.3.1 Biofilm formation on plastic films. The appearance of HDPE, PET and PVC films at each sampling point after submersion at the sea-end of Queen Anne's Battery Plymouth. (scale bar = 5 mm).....	68
Figure 3.3.2 Boxplots representing the biofilm quantification by crystal violet staining assay on the plastic films at each sampling point. (a) HDPE (b) PET (c) PVC (n=8). Time 0 represents the polymer without biofilm. ....	69
Figure 3.3.3 Scanning electron micrographs of microbial biofilm on plastic. Biofilm on HDPE films sampled after (a) control, (b) 3, (c) 6, (d) 9, (e) 12, (f) 18, (g) 30, (h) 42, (i) 56 -weeks exposure in marine water. Scale bar = 5 $\mu$ m. ....	72
Figure 3.3.4 Scanning electron micrographs of microbial biofilm on plastic. Biofilm on PET films sampled after (a) control, (b) 3, (c) 6, (d) 9, (e) 12, (f) 18, (g) 30, (h) 42, (i) 56 -weeks exposure in marine water. Scale bar = 5 $\mu$ m. ....	73
Figure 3.3.5 Scanning electron micrographs of microbial biofilm on plastic. Biofilm on PVC films sampled after (a) control, (b) 3, (c) 6, (d) 9, (e) 12, (f) 18, (g) 30, (h) 42, (i) 56 -week exposure in marine water. Scale bar = 5 $\mu$ m. ....	74
Figure 3.3.6 Bacterial community composition of plastics exposed to the marine environment and within surrounding seawater over time. Displayed are bacterial taxonomic classes with abundances of >0.1% in at least each sample at each sampling point. ....	75
Figure 3.3.7 Principle Coordinate Ordination (PCO) analysis. PCOs representing similarity of the bacterial communities based on Bray-Curtis similarity index, calculated from OTUs (>0.1%) data of the bacterial communities attached on plastics and within seawater samples. The symbols represent the sample types including seawater (SW) and plastics (HDPE, PET and PVC). The numbers indicate the time of sampling in weeks. Clusters represent samples sharing at least 50% similarity of their bacterial communities.....	77

Figure 3.3.8 Heatmap of 36 core OTUs relative abundances across all plastic samples at the beginning indicated as “early” (3-12 weeks), middle indicated as “intermediary” (18-30 weeks) and end indicated as “late” (42-52 weeks) of the exposure. Colour key represents the log-transformed relative abundances (in percentage). Samples were clustered hierarchically based on SIMPROR permutation tests and core OTUs were based on similarity index of association (average linkage). Red lines indicate clusters that are not significantly different ( $p < 0.05$ ). .....	80
Figure 4.2.1 Schematic illustration of the tensile stress test specimen. h: mean thickness, b: width, $\ell$ : total length, $\ell_o$ : gauge length. ....	90
Figure 4.3.1 Average monthly temperature ( $^{\circ}\text{C}$ ) and light intensity (lux) over the period of exposure experiment. The error bars indicate standard error of the mean. ....	93
Figure 4.3.2 Scanning electron micrographs showing the topography of HDPE films, sampled after (a) control, (b) 3, (c) 6, (d) 9, (e) 12, (f) 18, (g) 30, (h) 42, (i) 56 -week exposure in sea water. Scale bar = 5 $\mu\text{m}$ . ....	95
Figure 4.3.3 Scanning electron micrographs showing the topography of PET films, sampled after (a) control, (b) 3, (c) 6, (d) 9, (e) 12, (f) 18, (g) 30, (h) 42, (i) 56 -week exposure in sea water. Scale bar = 5 $\mu\text{m}$ . ....	96
Figure 4.3.4 Scanning electron micrographs showing the topography of PVC films, sampled after (a) control, (b) 3, (c) 6, (d) 9, (e) 12, (f) 18, (g) 30, (h) 42, (i) 56 -week exposure in sea water. Scale bar = 5 $\mu\text{m}$ . ....	97
Figure 4.3.5 Contact angle (CA) measurement for HDPE, PET and PVC films as a function of sampling time. (a) water contact angle; (b) formamide contact angle. The error bars indicate the standard error. ....	98
Figure 4.3.6 Tensile stress (MPa) of HDPE, PET and PVC films, exposed to seawater over the period of 56 weeks. The error bars indicate the standard error. ....	100
Figure 4.3.7 ATR-FTIR spectra of HDPE films. (a) Comparative spectra of HDPE outer surface at 0 (control), 3, 6, 9, 12, 18, 30, 42, and 56 -weeks of exposure to seawater, with the intensity of peaks recorded as arbitrary units (a.u.). For better visualisation, the ATR-FTIR spectra are enlarged at wavenumbers (b) 600-900, (c) 900-1400, (d) 1400-1600 and (e) 2600-3200. ....	103
Figure 4.3.8 ATR-FTIR spectra of PET films. (a) Comparative spectra of PET outer surface at 0 (control), 3, 6, 9, 12, 18, 30, 42, and 56 -weeks of exposure to seawater, with the intensity of peaks recorded as arbitrary units (a.u.). For better visualisation, the ATR-FTIR spectra are enlarged at wavenumbers (b) 650-1000, (c) 1000-1200, (d) 1200-1550 and (e) 1550-1850. ....	104
Figure 4.3.9 ATR-FTIR spectra of PVC films. (a) Comparative spectra of PVC outer surface at 0 (control), 3, 6, 9, 12, 18, 30, 42, and 56 -weeks of exposure to seawater, with the intensity of peaks recorded as arbitrary units (a.u.). For better visualisation, the ATR-FTIR spectra are enlarged at wavenumbers (b) 550-850, (c) 850-1200, (d) 1200-1800 and (e) 2750-3150. ....	105
Figure 4.3.10 Boxplots representing the carbonyl index (CI) measurements for HDPE, PET and PVC films as a function of sampling time. (a) HDPE; (b) PET; (c) PVC. ....	106



Figure 5.2.1 Experimental set-up for biodegradation test. (a) Schematic diagram of respirometric system components; (b) The original laboratory set-up of the respirometric system .....	117
Figure 5.3.1 Mixed bacterial growth on the surface of plastics. (a) HDPE, (b) PET and (c) PVC.....	121
Figure 5.3.2 Total carbon dioxide (CO <sub>2</sub> ) evolved from Blank, Cellulose, HDPE, PET and PVC treatment over a seven-month incubation in artificial seawater along with respective marine bacterial consortia. The shaded areas represent $\pm 1$ standard deviation of the mean. ....	123
Figure 5.3.3 Percentage of estimated carbon dioxide that was evolved from Cellulose, HDPE, PET and PVC treatment over a seven-month incubation in artificial seawater along with respective marine bacterial consortia. ....	124
Figure 5.3.4 Scanning electron micrographs showing the attached biofilm and topography of plastic films. (a) HDPE control, (b) HDPE after (t-210 days), (c) PET control, (d) PET after (t-210 days), (e) PVC control, (f) PVC after (t-210 days) of the respirometric test. Scale bar = 5 $\mu$ m.....	125
Figure 5.3.5 ATR-FTIR spectra of HDPE films. Comparative spectra of HDPE before (t-0) and after (t-210) days (with biofilm and cleaned) of the respirometric test. The intensity of peaks recorded as arbitrary units (a.u.). ....	127
Figure 5.3.6 ATR-FTIR spectra of PET films. Comparative spectra of PET before (t-0) and after (t-210) days (with biofilm and cleaned) of the respirometric test. The intensity of peaks recorded as arbitrary units (a.u.). ....	127
Figure 5.3.7 ATR-FTIR spectra of PVC films. Comparative spectra of PVC before (t-0) and after (t-210) days (with biofilm and cleaned) of the respirometric test. The intensity of peaks recorded as arbitrary units (a.u.). ....	128
Figure 5.3.8 Carbonyl indices (CI) of HDPE, PET and PVC films before (t-0) and after (t-210) the biodegradation test. **indicates significance p-value < 0.001, *indicates significance p-value < 0.05.....	129
Figure. Appendix D.1 Rarefaction analyses, indicating the $\alpha$ -diversity of bacterial communities in the seawater samples at the beginning (0-week) and end (56-week) of exposure experiment. ....	148
Figure. Appendix E.1 Rarefaction analyses, indicating the $\alpha$ -diversity of HDPE biofilm bacterial communities at the sampling period of 3, 6, 9, 12, 18,30, 42 and 56 weeks. ....	148
Figure. Appendix F.1 Rarefaction analyses, indicating the $\alpha$ -diversity of PET biofilm bacterial communities at the sampling period of 3, 6, 9, 12, 18,30, 42 and 56 weeks .....	149
Figure. Appendix G.1 Rarefaction analyses, indicating the $\alpha$ -diversity of PVC biofilm bacterial communities at the sampling period of 3, 6, 9, 12, 18,30, 42 and 56 weeks. ....	149

# List of Tables

Table 1.4.1 List of microorganisms isolated from the marine environment with the capability of degrading various plastics.....	26
Table 2.2.1 Name and properties of the plastics used in the Study .....	30
Table 2.3.1 PERMANOVA test results of Bray-Curtis dissimilarities of bacterial community structure in relation to control vs plastic treated NSW. Tr = Treatment; Ti = Time; df = degree of freedom; SS = sum of squares; Pseudo-F = F value by permutation, boldface indicates statistical significance at $P(\text{perm}) < 0.05$ , p-values based on 999 permutations, P (MC) = p-values using Monte-Carlo tests, P (PERMDIP) = p-values from PERMDISP tests, calculated to centroids.....	43
Table 2.3.2 Identification of T-RFLP fragments using MiSeq sequencing .....	45
Table 2.3.3 Results of DISTLM marginal tests. Results of marginal tests using a distance-based linear model (DISTLM) quantifying the relative contribution of chemical variables (DIC, DOC, TN and pH) on seawater bacterial community in all plastic treatments. Variables with significant values (P) were listed in bold. Prop.Var. the proportion of explained variance of corresponding seawater variable.....	50
Table 2.3.4 Results of the DISTLM using 'Best' selection procedure showing the correlation coefficients between the Best all possible combinations of chemical parameters (marked by '✓') and the variability of seawater bacterial community in all plastic treatments. $R^2$ values reported for corresponding significant variables and Overall Best model $R^2$ values for all variables. ....	51
Table 3.3.1 Mixed factorial repeated measures ANOVA results of biofilm (OD 595nm) data in relation to plastic-type and exposure time. SS = Type III sum of squares; df = degree of freedom; F = F-value; p = significance value .....	70
Table 3.3.2 SIMPER analysis of bacterial communities, presenting the total similarity within and dissimilarity between the seawater and plastics attached communities. Av.Si%: average percentage similarity within all sampling points, Av.δi %: average dissimilarity between the seawater and plastic attached bacterial communities.....	78
Table 4.2.1 Wavenumbers selected as reference peak area for HDPE, PET and PVC to measure the carbonyl index.....	92
Table 4.3.1 Mixed factorial repeated measures ANOVA results of H <sub>2</sub> O and formamide contact angle (CA) data in relation to plastic-type and exposure time. ....	99
Table 4.3.2 Mixed factorial repeated measures ANOVA results of tensile stress data in relation to the plastic-type and exposure time. ....	100
Table 5.2.1 The total organic carbon and the wet and dry mass of the test materials used for the study. ....	119
Table 5.3.1 The taxonomical identification of marine bacteria isolated from the surface of plastics after the enrichment procedure.....	122

Table. Appendix A.1 SIMPER analysis of bacterial communities, presenting the total similarity within all sampling points and dissimilarity between the control and plastics treated communities. Av.Si%: average percentage similarity within all sampling points, Av.δi %: average dissimilarity between the control and plastics treated bacterial communities..... 145

Table. Appendix B.1 PERMANOVA pair-wise tests of bacterial communities within plastic treatments and control based on Bray-Curtis similarities index. Significance results (p (perm) < 0.05) are in bold..... 146

Table. Appendix C.1 PERMDISP pair-wise tests of bacterial communities within plastic treatments and control based on Bray-Curtis similarities index. Significance results (p (perm) < 0.05) are in bold..... 147

# List of Abbreviations

ANOVA	Analysis of variance
ASTM	American Society for Testing and Materials
ASW	Artificial seawater
BPA	Bisphenol A
CA	Contact angle
CH <sub>4</sub>	Methane
CI	Carbonyl index
CO <sub>2</sub>	Carbon dioxide
CTAB	Hexadecyltrimethylammonium bromide
CV	Crystal violet
dbRDA	Distance-based redundancy analysis
DDT	Dichlorodiphenyltrichloroethane
DIC	Dissolved inorganic carbon
DISTLM	Distance-based linear models
dNTP	Deoxynucleotide triphosphate
DOC	Dissolved organic carbon
DOM	Dissolved organic matter
EPS	Extracellular polymeric substances
FEG-SEM	Field emission gun scanning electron microscopy
FTIR	Fourier transform infrared spectroscopy
GLM	Generalised linear model
HCl	hydrochloric acid
HDPE	High-density polyethylene plastic
IR	Infrared radiation
KNO <sub>3</sub>	potassium nitrate
LDPE	Low-density polyethylene
N <sub>2</sub>	Molecular nitrogen
NaCO <sub>3</sub>	Sodium carbonate
NaHCO <sub>3</sub>	Sodium hydrogen carbonate
NGS	Next-generation sequencing
NO	Nitrogen monoxide
NPOC	Non-purgeable organic carbon
NSW	Natural seawater
OD	Optical density
OsO <sub>4</sub>	Osmium tetroxide
OTUs	Operational taxonomic units
PAHs	Polycyclic aromatic hydrocarbons

PCBs	Polychlorinated biphenyls
PCO	Principal coordinates analyses
PCR	Polymerase chain reaction
PERMANOVA	Permutational Multivariate Analysis of Variance
PERMDISP	Permutational analysis of multivariate dispersions
PET	Polyethylene terephthalate
PHB/PHV	Polyhydroxybutyrate/Polyhydroxyvalerate
PLA	Polylactic acid
POM	Particulate organic matter
POPs	Persistent organic pollutants
ppm	Parts per million
PS	Polystyrene
PU	Polyurethane
PVC	Polyvinyl chloride
PVOH	Poly (vinyl alcohol)
RDP	Ribosomal database project
ROOH	Hydroperoxides
rRNA	Ribosomal ribonucleic acid
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SIMPER	Analysis of dissimilarity, statistical test
T-RF	Terminal restriction fragment
T-RFLP	Terminal restriction fragment length polymorphism
TC	Total carbon
TN	Total nitrogen
TOC	Total organic carbon
TOC-V	Shimadzu Total Organic Carbon VCSN analyser
UV	Ultraviolet

# **Chapter 1: Introduction**

## **1.1 Importance of the Research Area**

### **1.1.1 Plastic Entanglement and Ingestion**

Marine plastic debris is the cause of numerous ecologically damaging and hazardous effects (Derraik, 2002). It poses a direct threat either through entanglement, which leads to trapping, injury, restrictions to feeding and growth of marine wildlife, or by ingestion; causing clogging of gills, gastrointestinal blockage, reduced secretions of gastric enzymes and steroid hormones (Azzarello and Van Vleet, 1987, Beck and Barros, 1991, Campagna et al., 2007, Dau et al., 2009, Cedervall et al., 2012, Vélez-Rubio et al., 2013, Browne et al., 2013, Oliveira et al., 2013, Wright et al., 2013a). These hazards may ultimately result in body deformations, starvation, drowning, and an inability to escape predators.

Entanglement has been known to affect 344 species to date and often results in the death of the affected animal (Kühn et al., 2015, Gall and Thompson, 2015). Research by Kühn et al., (2015) found affected species included 100 % of marine turtles, 67 % of seals, 31 % of whales and 25 % of seabirds sampled. In addition, 92 species of invertebrates and 89 fish species have also become entangled with marine plastic debris (Kühn et al., 2015). Entanglements usually occur with discarded or derelict plastic ropes and fishing nets, commonly known as “ghost” nets. It has been demonstrated that 5000 to 15000 turtles were caught by these ghost nets on Australia’s northern shore within one year (Wilcox et al., 2015a). Similarly, another study along the north-western coast of the United States estimated that a single discarded net or plastic rope caught one fish and a seabird in every three and five days respectively, as well as two invertebrates per day (Gilardi et al., 2010). Entanglement can also be caused by strapping, packaging bands or balloon strings and the other parts of debris forming entangling loops (Fowler, 1987, Gall and Thompson, 2015).

Plastic ingestion is a widespread phenomenon and reported across various marine habitats, trophic levels and geographic regions (Provencher et al., 2017). Data on plastic debris ingestion is increasing due to recent close tracking of plastic pollution and the investigation of wide-ranging marine organisms (van Sebille et al., 2015). The most recent estimates show

that 233 marine species are affected by plastic debris due to ingestion, which includes sea turtles, seabirds, cetaceans and manatees (Kühn et al., 2015, Gall and Thompson, 2015, Ryan, 2015). Species in these studies with the highest number of individuals ingesting plastic debris were sea turtles (100 %), whales and seabirds (59 % each), and seals (36 %) (Kühn et al., 2015). Moreover, ingestion of plastics has also been reported in 92 fish and 6 invertebrates (Kühn et al., 2015, Wilcox et al., 2015b). These marine species ingest plastics through either opportunistic feeding or mistaking plastic for food/prey, following visual or olfactory signals (Law, 2017). For instance, sea turtles mistake clear plastic bags for jellyfish, which results in the obstruction of their gut and internal injury after ingestion (Schuyler et al., 2014) and reproductive impairments (Plot and Georges, 2010). Some seabirds that have evolved olfaction are attracted towards the infochemicals produced by their phytoplankton prey. These chemicals are easily absorbed by floating plastics and therefore mistakenly consumed by seabirds (Savoca et al., 2016).

In contrast to entanglement, no specific form or plastic material is generally associated with ingestion, however, the size of the ingested items is limited by the size of the ingesting organism. For example, small plastic particles and fibres have been detected in small filter-feeders such as mussels and oysters (Van Cauwenberghe et al., 2013) and suspension-feeding barnacles (Goldstein and Goodwin, 2013). Larger debris items (e.g. cigarette box wrapping and potato chip bags) have been found in the stomachs of large pelagic fish (Jackson et al., 2000) and very large items including: 4.5 m of hose; 9 m of rope; two flowerpots and large amounts of plastic sheeting were found to be ingested by sperm whale (de Stephanis et al., 2013).

### **1.1.2 Plastic Toxicity**

During the production of plastics, various chemicals are added to polymer resins which alter the physical properties of the product (Kitada et al., 2008). These additives include plasticisers to promote material flexibility, inorganic fillers (e.g. carbon and silica fillers) that strengthen the material, as well as chemicals that act as flame retardants and thermal/ultraviolet stabilizers. These additives are of environmental concern as they are not chemically bound to the plastics and therefore they leach into water, sediments and even into an organism (Teuten et al., 2009, Barnes et al., 2009, Lithner et al., 2011). Though many additives have been phased out of plastic production, their legacy remains in landfills and on sea beds (Worm et al., 2017). Frequently used additives including bisphenol A (BPA); phthalates; polybrominated diphenyl ethers; and alkylphenols, which are hazardous to biota.

They can act as endocrine-disrupting chemicals which can compete with, or disrupt the synthesis of endogenous hormones (Talsness et al., 2009). High concentrations of these additives have been found in plastic fragments sampled both at remote and urban beaches, as well as in those floating in the open ocean (Hirai et al., 2011).

Marine plastic debris not only carries hazardous additives, but can also act as a vector for hydrophobic contaminants (e.g. polycyclic aromatic hydrocarbons (PAHs) and dichlorodiphenyltrichloroethane (DDT)) which are adsorbed from the surrounding water (Frias et al., 2010). These contaminants belong to a group of chemicals known as persistent organic pollutants (POPs) that are resistant to environmental degradation. DDT previously isolated from marine plastics is known to have a range of impacts on marine animals and its accumulation has been observed in many marine organisms (de Azevedo e Silva et al., 2007). Polychlorinated biphenyls (PCBs) is a PAH that contains chlorine and a bisphenol (a pair of benzene rings) and has been observed in a variety of marine fauna (Besseling et al., 2013). Various PCBs differ in toxicity but have been observed to result in high mortality even after a single exposure (Faroon and Ruiz, 2016). The adsorption of POPs to plastic particles has been widely reported (Karapanagioti and Klontza, 2007, Hirai et al., 2011, Rochman et al., 2013a, Koelmans et al., 2013), with the adsorption rate dependent upon the type of plastic, its chemical and physical properties, surface area and the chemical exposure throughout the particle's drift history (Rochman, 2015).

Adsorbed chemicals on plastic pollution can transfer to marine organisms via leaching and ingestion and cause reduced feeding, reproductively, as well as changes in enzyme and immune functions (Tanaka et al., 2013, Rochman et al., 2013b, Avio et al., 2015, Bejgarn et al., 2015). Moreover, the transfer of these environmental pollutants to marine organisms can lead to significant bioaccumulation and thus potentially pose a direct threat to human health (Hirai et al., 2011).

### **1.1.3 Economic Effects**

One of the repercussions of marine plastic debris is the economic impact which increases the costs of coastal and maritime activities (Newman et al., 2015).. Economic costs of marine plastic debris cannot be easily measured as most of the environmental and economic impacts are intertwined (McIlgorm et al., 2011, Niaounakis, 2017). However, some of the impacts are direct and easier to measure in economic terms, for instance, the costs associated with the vessel downtime due to its propeller becoming entangled with plastic debris. Others



indirect effects can be more complex, such as the costs arising from ecosystem deterioration and human food chain contaminations (McIlgorm et al., 2011, Newman et al., 2015).

The main sectors which are affected by marine plastic pollution are fisheries, transportation, coastal local authorities and tourism (Newman et al., 2015). The fishing industry is usually considered a source of marine plastic, but it is also the most affected sector (economically) by plastic pollution in the oceans. The economic losses within this industry are associated with the repairing and cleaning of plastic entangled vessels and replacing the damaged or torn nets. In Britain, these losses cost the local fishing industry between \$15.5 million to \$17.2 million every year, equivalent to 5 % of the total revenue of affected fisheries (Mouat et al., 2010). Similarly, the shipping industry also faces economic impacts due to either entrapment or collision with bulky marine debris, thus results in high repair and labour costs at marinas and harbours. It has been estimated that it costs \$3.1 million per year to remove marine debris at the ports of the UK (Mouat et al., 2010).

Coastal local authorities are affected economically by marine debris mainly due to the clean-up costs of beaches and waterways (Newman et al., 2015). The annual clean-up cost for all the coastal authorities in the UK was estimated to be between \$24 million to \$25.3 million in 2010, equivalent to \$189,800 per local authority (Mouat et al., 2010). Moreover, the marine debris wash-ups and lack of cleanliness decrease beach attendance and revenue from tourism. For example, a study in South Korea calculated the annual loss of \$40-\$51 million due to decreased beach attendance by 63 % in 2011 (Jang et al., 2014). The overall economic damages due to marine plastic pollution reduce 1-5 % of the benefits derived from maritime activities which are valued to be up to \$2.5tn a year (Beaumont et al., 2019).

There are clear economic and environmental consequences of plastic pollution in the World's Oceans. Further investigating the consequences of plastic, therefore, warrants scientific attention. In particular, the role microbes play in the colonisation and the ultimate breakdown of plastics has only recently been explored (Zettler et al., 2013). This research area will, therefore, form the basis of this thesis.

## **1.2 Objectives**

The benefits of plastic and the exponential increase in production indicates that polymers will continue to play an integral part in human society. An unfortunate consequence of this trend, however, is that large quantities of plastic material will continue, often inadvertently,

to enter the World's Oceans. The primary objective of this research is to understand what happens to this pollution in terms of microbial colonisation and to what extent plastic in the World's oceans is being broken down (physically and biologically). Moreover, this research investigates the impact that plastic pollution can have on the inherent chemical and biological characteristics of seawater, and whether any subsequent changes could have implications for ocean biogeochemical cycling. To address these questions a combination of laboratory simulations and natural fieldwork experiments were undertaken.

The structure of this thesis is as follows:

Chapter 1: This Chapter provides justification of the research area and question. It contains a scientific literature review that focuses on the interactions between marine microorganisms and plastics, and the degradation of plastics in the marine environment.

Chapter 2: This Chapter is a laboratory time-series investigation into the effects of plastic pollution on the microbial community structure and diversity within seawater. It details the changes in microbial and physio-chemical properties of seawater that occur when plastic is added to it.

Chapter 3: This Chapter investigates microbial community (biofilm formation and progression) succession on plastic surfaces suspended in natural seawater over a one-year period. It details temporal variations and identifies common genera within the plastic colonising bacterial assemblages.

Chapter 4: This Chapter is the physical sciences companion to Chapter 3: and investigates the mechanic stability and chemical composition of plastic from a long-term *in situ* exposure study.

Chapter 5: This Chapter isolates bacterial consortia from seawater based on the ability to utilise plastics as a carbon source. The bacteria are further enriched and used to investigate the degradation of plastics in a simulated seawater environment, as well as reporting any resulting changes in the physicochemical properties of test plastics.

Chapter 6: This Chapter provides a synthesis of all the results and provides a critical assessment of the experimental limitations. It also provides suggestions for future work.

The following section (1.3) details the scientific concepts and background literature that underpins the data Chapters (Chapter 2: p27, Chapter 3: p60, Chapter 4: p86, Chapter 5: p111, of this thesis.

## **1.3 Literature Review**

### **1.3.1 Production**

Plastics are long-chain synthetic polymers that have a high molecular mass and primarily consist of carbon, hydrogen and oxygen atoms. The term plastic is derived from the Greek word “plastikos” meaning mouldable into any shape (Fried, 1995). Plastics were developed in the early twentieth century to replace depleted natural resources. The world’s first commercially important synthetic plastic, Bakelite, was invented by Leo Baekeland in 1907 (Baekeland, 1909). It was formed by mixing two common chemicals, phenol and formaldehyde, under high pressure and temperature (Baekeland, 1909). The resulting plastic material opened the door to the modern “Plastic Age” and spurred the growth of a worldwide multi-billion industry that set out to transform every aspect of human material consumption (Worm et al., 2017).

Plastics are mainly derived from petrochemicals and are formed by a range of chemical processes (Shimao, 2001). They are produced by the polymerisation reaction, a process of chemically joining the monomer units to form long-chain molecules (McKeen, 2008). There are two mechanisms for polymerisation; addition and condensation. During addition polymerisation reactions, the monomers adhere to each other in the presence of a catalyst, peroxide or other initiator molecule. This reaction usually begins with the breakdown of double bonds between the carbon atoms of unsaturated monomers which forms a free radical. The radical then causes one monomer to bond with the adjacent monomer and creates new radicals. The repetitive formation of free radicals and joining of monomers creates long-chain polymers, with no by-products formed during this reaction (Jenkins and Mills, 2005). By contrast, condensation polymerisation – a process of joining monomers through reactive or functional groups – results in the formation of a by-product, such as water. This reaction requires two chemically distinct monomers to join alternatively. The subsequent addition of monomers to the reactive group is known as step-growth, which results in the formation of dimers, tetramers and other oligomers (Jenkins and Mills, 2005). Examples of the most common plastics along with their chemical structure and functional groups are shown in Figure 1.3.1. These functional groups define the overall strength and ease at which the polymers can be degraded (Jenkins and Mills, 2005).

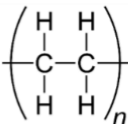

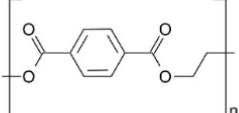

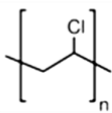

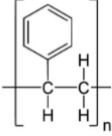
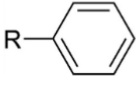
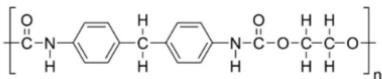

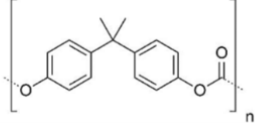
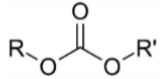
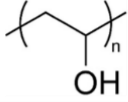

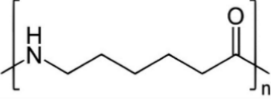

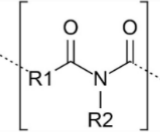
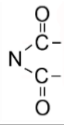
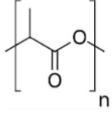

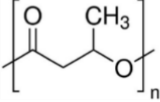
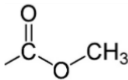
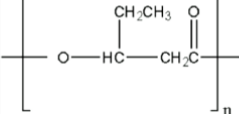
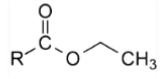
Plastic Type	Structural formula	Functional group
<b>Fossil-based</b>		
Polyethylene (PE)		Alkene 
Polyethylene terephthalate (PET)		Ester 
Polyvinyl chloride (PVC)		Alkyl halide 
Polystyrene (PS)		Phenyl 
Polyurethane (PU)		Urethane 
Polycarbonate (PC)		Carbonate 
Polyvinyl alcohol (PVOH)		Hydroxyl 
Nylon		Amide 
Polyimide (PI)		Imide 
<b>Bio-based</b>		
Poly lactic acid (PLA)		Ester 
Poly (3-hydroxybutyrate) (PHB)		Methyl ester 
Poly (3-hydroxyvalerate) (PHV)		Ethyl ester 

Figure 1.3.1 Chemical structures of some common plastics and their functional groups

Plastics are low-cost, lightweight, durable materials, with high electrical and thermal insulation properties. The diversity of plastics and their versatile properties have made them ubiquitous in all aspects of modern daily life including: packaging; clothing; transportation; buildings; energy savings and various other societal benefits (Andrady and Neal, 2009). Every year new applications are developed and the substitution of other materials with plastic is still expanding in many sectors (e.g. drug carriers, engineering tissues) (Worm et al., 2017). The worldwide production of plastics has increased from 0.5 million metric tons per year ( $\text{Mt year}^{-1}$ ) in 1950 to 348  $\text{Mt year}^{-1}$  in 2017 (Figure 1.3.2 a) (Plastics Europe, 2018). The production of plastics in 2017 was centred in Asia (50.1 % of the global output), with China being the world's largest producer (29.4 %), followed by Europe and North America, each contributing 18.5 and 17.7 %, respectively (Plastics Europe, 2018). Single-use packaging sector utilises about 40 % of the total plastic produced in Europe, followed by consumer items (22 %); building and construction materials (20 %), automotive (10 %), electronics (6 %), and the agriculture sector (3 %) (Figure 1.3.2 b).

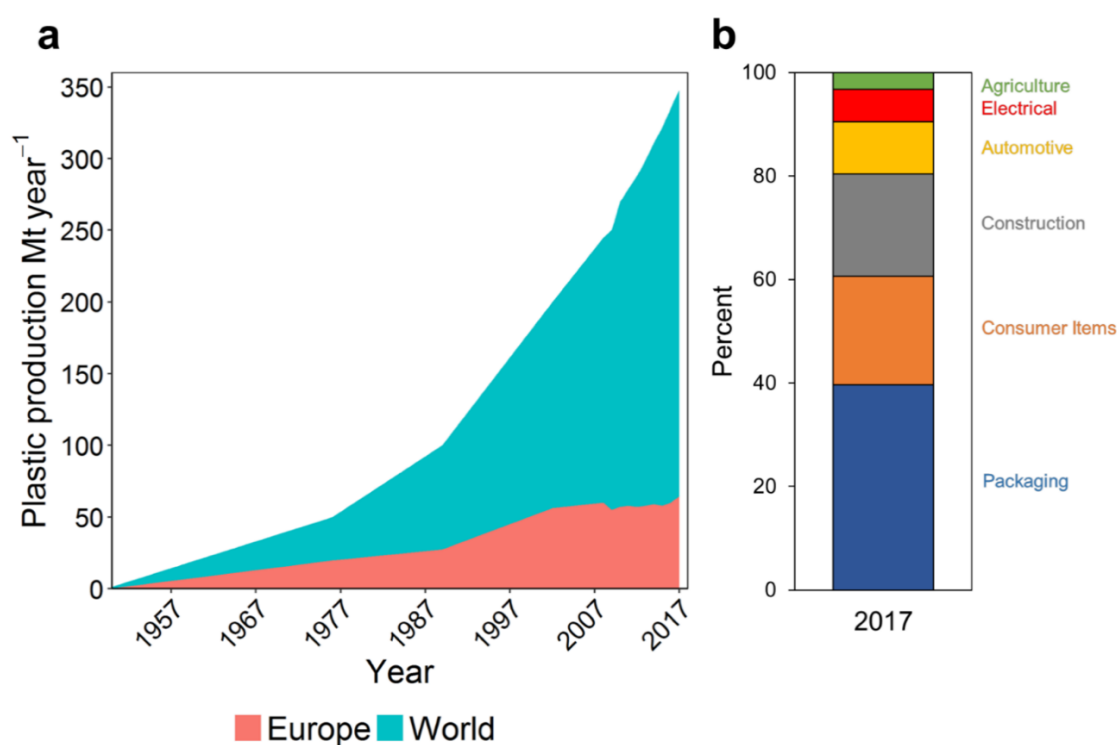


Figure 1.3.2 Trends of global plastics production. (a) Worldwide and European plastics production in million metric tons per year ( $\text{Mt year}^{-1}$ ) from 1950 to 2017. (b) Plastic applications patterns in percent during 2017. Data compiled from Geyer et al. (2017) and Plastics Europe (2018).

### 1.3.2 Origin and Levels of Plastic Pollution in the Marine Environment

A substantial quantity of plastic waste ends up as litter in the marine environments and can be categorised according to its origin, either from the sea- or land-based sources. (Depledge et al., 2013, Brouwer et al., 2017, Hahladakis et al., 2018). Plastic waste from land-based

sources accounts for 80 % of the total marine plastic debris and enters the world's oceans via wastewater drainage, river systems, terrestrial winds, catastrophic events (tsunami and flooding) and human negligence (Derraik, 2002, Hammer et al., 2012). The remaining 20 % of marine plastic debris comes from ocean-based sources, which includes ships, offshore facilities, fishing, recreational and research vessels (Li et al., 2016, Law, 2017).

Figure 1.3.3 shows the potential pathways and sources of plastics into the marine environment. The first entry point of plastic waste into the marine environment is the accidental spillage or mishandling of resin pellets which are used as feedstock for the manufacturing of plastic products. These spilt pellets may directly enter the oceans through rivers and coastlines or be washed into wastewater drains or storm-water (US-EPA, 1992). Resin pellets were among the first items of plastic debris reported in the ocean (Carpenter et al., 1972) and have been discovered on beaches and at sea all over the world (Hirai et al., 2011). The abundance of pellets has steeply declined since the 1980s (Van Franeker et al., 2011), which was attributed to the prevention measures recommended to the plastics industries by Governments (e.g. US-EPA, 1992). These measures included: instalment of pellet containment systems; improved clean up procedures, employee training and inspection of containers before loading and after offloading of pellets (US-EPA, 1992).

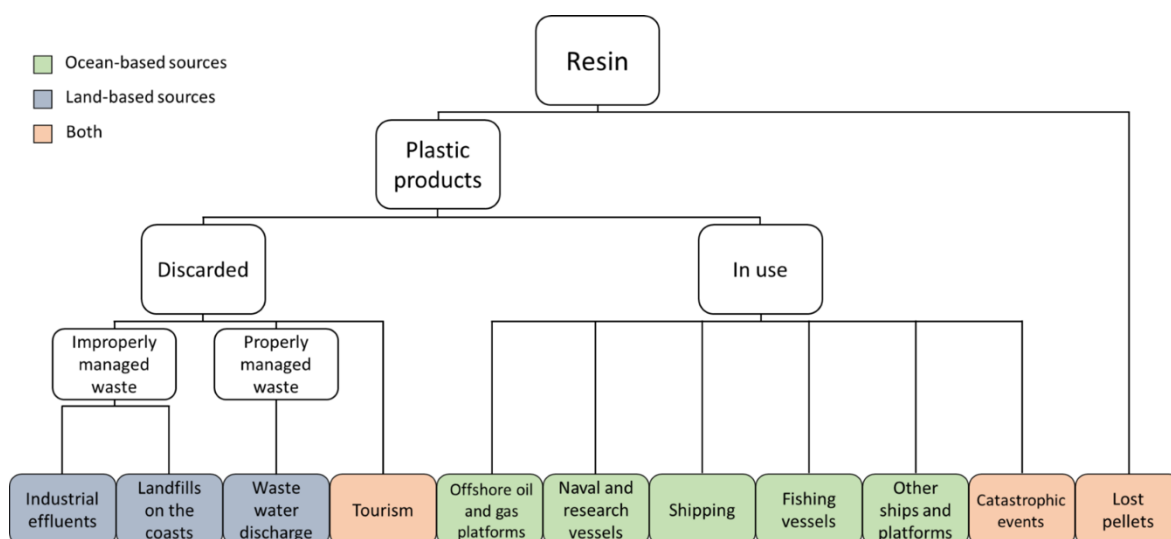


Figure 1.3.3 The pathways of plastic waste into the marine environment. The direct sources of plastic waste from sea-based activities are shown in green shading, from land-based activities in purple, and from either maritime or land activities in orange. Redrawn and adapted from (Hammer et al., 2012, Law, 2017).

Once the resin pellets are converted into plastic products, they can enter the environment either accidentally during use or upon disposal as waste. Discarded plastic products are either managed through waste management infrastructure or dumped in uncontained/open landfills (poorly managed waste). Similarly, treated wastewater discharge is also classified as managed waste; however, plastic microbeads (commonly known as microplastics, size < 5

mm) which are used as irregular abrasives in many cosmetics, as well as fibres released from synthetic clothing upon washing (Browne et al., 2011, Napper and Thompson, 2016), can enter household wastewater. The capture of these particles in wastewater treatment plants (i.e. before the effluent is discharged to the environment) depends on the specific treatment process. High capture rates (> 95 %) of plastic particles were found by previous studies of wastewater treatment plants (Magnusson and Norén, 2014, Carr et al., 2016). However, there are large quantities of effluents processed through such treatment plants every day, therefore even a low loss rate results in detectable concentrations in the environment (Browne et al., 2011, Eriksen et al., 2013). In addition, catastrophic events such as floods, hurricanes and tsunamis result in the unintentional loss of in-service plastic products in the marine environment. Such losses also occur when cargo or gear is lost during marine transportation or use (Figure 1.3.3). Presently, the total amount of plastics entering the world's oceans due to the catastrophic events or the marine activities is not known (Law, 2017).

Improperly managed plastic waste on land is the only major source of ocean plastics that have been estimated globally (Jambeck et al., 2015). The analyses by Jambeck et al. (2015) used global data on solid waste generation, population density, waste composition, disposal and economic status in 192 coastal countries to calculate the amount of plastic waste produced. It also included the amount that is uncontained due to open dumping and littering. The calculation of annual input of plastic waste into the oceans was based on the inland waste generation by the population living within 50 kilometres of the coast which could be transported to the oceans. It has been estimated that 275 Mt of plastic waste was generated in 2010 by these coastal countries, of which 4.8–12.7 Mt ended up in the oceans, equivalent to the 1.7–4.6 % of the total plastic waste produced by these countries. Furthermore, Jambeck et al. (2015) also estimated that the amounts of plastic waste entering the marine environments from the land would increase by an order of magnitude by 2025 if there are no improvements to current waste management infrastructure.

### **1.3.3 Degradation of Plastics in the Marine Environment**

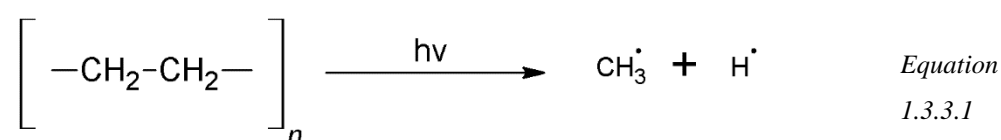
In the natural environment, carbon-based materials (such as plastic) are broken down by physicochemical processes and biological organisms to produce gas (carbon dioxide (CO<sub>2</sub>) in aerobic and methane (CH<sub>4</sub>) in anaerobic environments), water and biomass. Degradation is a chemical or physical change in the plastics exposed to environmental factors, such as sunlight, temperature, moisture, mechanical processes or microorganisms (Shah et al., 2008). It is a partial or complete breakdown of polymers under the influence of one or more

such environmental factors. These factors either accelerate or decelerate the degradation process within diverse marine environments, including beaches, water surface, the water column and seafloor (Niaounakis, 2017). There are five degradation processes by which plastics can degrade in marine environments.

### 1.3.3.1 Photodegradation

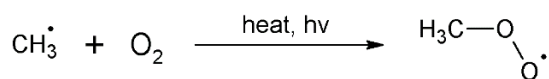
Photodegradation is a process that causes the molecular weight of polymers to reduce when exposed to sunlight, resulting in embrittlement and degradation of the polymers (Singh and Sharma, 2008). At ambient conditions, this is considered as one of the primary sources of polymer degradation (Gijsman et al., 1999). Photodegradation occurs through the absorption of ultraviolet (UV) rays by the units or groups that form the chemical structure of polymers, or through coloured impurities called chromophores. These radiations include UV-A (~315–400 nm) and UV-B rays (~280–315 nm), and when plastic is exposed to them it reduces the molecular weight of the material (Shah et al., 2008).

Photodegradation is induced by two distinctive processes which rely on absorption of UV radiations. (1) Photolysis of UV susceptible groups within plastic materials, (2) the utilisation of photo-initiators for the oxidation of the plastic substrate (Scott, 1976, Nakamura et al., 2006). When exposed to sunlight as an initiator, free radicals are formed within the polymer via C-H bond dissociation in the polymer chain (Equation 1.3.3.1).



The extent of this chemical reaction relies on the amount of ultraviolet rays (<350nm) to which plastic is exposed. However, once the reaction is triggered and the free radicals have been produced, hydroperoxides (ROOH) are generated due to the presence of oxygen (Equation 1.3.3.2) (Wypych, 2013).



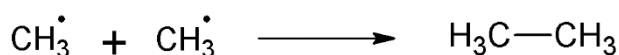


Equation

1.3.3.2



Various decomposition products including ketones and aldehydes are produced by the dissociation of hydroperoxides. The occurrence of carbonyl groups can be utilised as a degradation chemical index in a degraded polymer (Oliani et al., 2007). Although the reaction is initiated by UV exposure, once free radicals are formed, they continue to react through propagation reactions long after the initial UV exposure has ended. However, the reaction between free radicals results in the termination of the propagating free radicals (Equation 1.3.3.3).



Equation

1.3.3.3

The development of free radicals and propagation reactions do not have deleterious effects on polymer properties, as they do not cause significant alterations in the long-chain molecular structure of the polymer. Photodegradation of the polymer properties occurs due to the production of highly unstable free radicals, which readily undergo chain scission reactions (i.e. the random breaking of the carbon backbone of the polymer into fragments). This process eventually results in the creation of two smaller chains of polymers, one of which acts as free radical and have the ability to conduct further reactions (Wypych, 2013).

Degradation rates of plastics within marine environments are different due to variances in solar irradiation and temperature. Plastics materials lying on beaches go through rapid photodegradation as they are subjected to direct sunlight that results in UV-B exposure in combination with higher temperatures (Andrady, 2011). Plastics also tend to absorb infrared radiations, especially darker coloured polymers, which causes their surface temperature to rise. This “heat build-up” phenomenon promotes faster photodegradation of plastic materials (François-Heude et al., 2014). The photodegradation of polyethylene netting and twine (Meenakumari et al., 1995), fishing gear (Al-Oufi et al., 2004) and nylon monofilament

(Thomas and Hridayanathan, 2006) in supralittoral and intertidal zones have been previously reported.

The photodegradation of plastic materials floating in the surface water of the world's oceans is distinctly lower when compared to plastic pollution from coastal and terrestrial environments (Andrady, 2015b). There are two main reasons for this measured difference:

1. Lower (seawater temperature) plastic surface temperatures reduce the photo-oxidative degradation process.
2. Various marine species are involved in the fouling of plastics surfaces which makes them opaque. The thick foulant opaque layer guards the plastics against solar irradiation especially UV-B and thus prevents the oxidation process (Andrady, 2011).

Photodegradation is not measured beyond the photic zone in the oceans due to the limitation of light penetration. Moreover, the cold, deep anoxic water has little to no potential of oxidative plastic degradation. This is also true for marine sediments where plastics can accumulate (Thompson et al., 2004, Browne et al., 2010, Cole et al., 2011).

### **1.3.3.2 Thermal degradation**

Thermal degradation causes changes in the chemical structure of plastics (Pielichowski and Njuguna, 2005). This degradation process alters the entire plastic structure rather than just the surface and results in the loss of tensile strength, molecular weight changes, reduced durability, embrittlement, changes in crystallinity, modification in colour, chalking and cracking (Singh and Sharma, 2008, Olayan et al., 1996). Under ambient conditions, photo- and thermal degradations are similar and are classified as oxidative degradation. The main difference between the two is the sequence of initiation steps which leads to the auto-oxidation of plastics. Also, thermal reactions occur throughout the bulk of the polymer sample, whereas photodegradation reactions occur only on the surface (Tyler, 2004).

Thermal degradation of polymers occurs through random and chain degradation (depolymerisation reaction) initiated by thermal and UV light. When the temperature is high enough to cause bond cleavage, plastic degradation follows three key pathways: (1) side-group elimination, (2) random scission and (3) depolymerisation (Cooper, 2012). The side-group elimination pathway takes place in two stages. The initial step involves the omission of side groups that are attached to the backbone of the polymer and thus results in the formation of unsaturated chain structure. This unstable poly-unsaturated macromolecule

then goes through further degradation reactions such as scission process and turns into smaller fragments (Pielichowski and Njuguna, 2005).

Random scission of polymers occurs through the formation of free radicals alongside the polymer structure resulting in fragmentation. This mechanism results in the production of small repeating oligomers of varying chain length depending upon the number of carbons in the chain and the presence of a terminal free radical (Figure 1.3.4) (Webb et al., 2013). Furthermore, the depolymerisation pathway utilises the free radicals which are formed along the backbone of the polymer during the scission mechanism to produce small unsaturated molecules (Beyler and Hirschler, 2002). These unsaturated molecules undergo further breakage to monomers or co-monomers by continuous formation of free radicals. Depolymerisation of plastics occurs under elevated temperature condition which does not need to be started at the terminal end of the polymer, instead, it initiates at the weak links within the polymer structure (Singh and Sharma, 2008). Polymers that do not undergo depolymerisation are generally broken down into fragments by thermal stresses (Villetti et al., 2002).

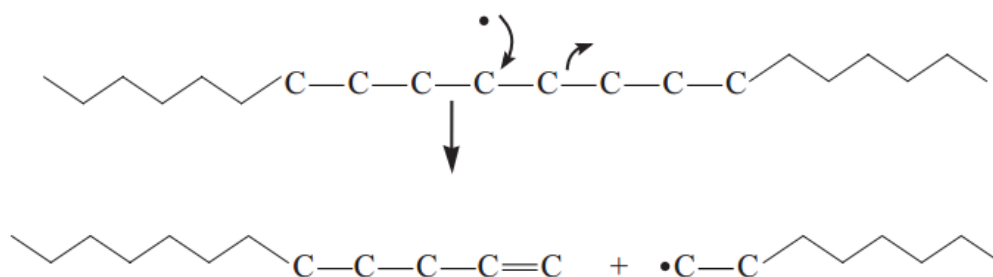


Figure 1.3.4 Random Scission mechanism for thermal degradation of plastics

In this section, the focus is on thermal degradation of plastics in the marine environment. Although plastics can undergo thermal degradation during product use and mechanisms for such polymer degradation has been reviewed elsewhere (Singh and Sharma, 2008, Ray and Cooney, 2018). In the natural environment, thermal degradation of plastics can occur, but photo-oxidation initiated by UV radiations and hydrolysis are more common under ambient conditions. In particular, the low temperatures typical of the marine environment mean that plastics degrade primarily by slow, photo-oxidative degradation (Andrady, 2011, Chamas et al., 2020). When a polymer sinks below the upper regions of the ocean with limited sunlight, it obviously cannot undergo photodegradation. Under such conditions, slow thermal oxidative degradation and hydrolysis may occur together, or sequentially (Chamas et al., 2020).

In a marine environment, the thermal degradation of plastics occurs along with photodegradation, hydrolysis, and biodegradation (Andrady, 2011). The plastic materials with carbon-carbon backbone (e.g. PE, PP, PVC) are potentially susceptible to thermal oxidation while plastics with heteroatoms in their main chain (e.g. PET, PU) are primarily degraded by slow photo-oxidation in low temperature marine environment (Venkatachalam et al., 2012, Gewert et al., 2015). In seawater, due to naturally occurring temperature gradients, thermal degradation is highest nearer coastal areas and is lowest in the open seas and at the bottom of the ocean (Andrady, 2011, Andrady, 2015b). The “heat build-up” phenomenon (see Section 1.3.3.1) can aid the thermal degradation process of plastics in the marine environment, consequently, plastic on beaches degrades at higher speeds (Cooper, 2012). The “heat build-up” phenomenon, however, is negated in seawater (François-Heude et al., 2014). Moreover, the lower temperatures of world’s oceans (average maximum temperature 17 °C) along with decreased oxygen and UV levels, reduces the degradation of plastics in seawater considerably (Webb et al., 2013, Gewert et al., 2015).

#### **1.3.3.3 Hydrolysis**

Hydrolysis is the cleavage of chemical bonds within the polymer structure by reaction with water (He et al., 2004, Tsuji and Miyauchi, 2001). This reaction occurs essentially in polymers that absorb moisture and have water-sensitive functional groups in their structural backbone. Polymers that have hydrolysable covalent bonds are more susceptible to hydrolysis; this includes polyethers, polyesters, polyamides, polycarbonates, and polyanhydride. The rate of hydrolysis can vary and is dependent upon water activity, which is the measure of free water and is defined as the ratio between the water vapour pressure of the solution and of free water under the same conditions (Koop et al., 2000). Moreover, pH, temperature, type of functional group and time are the other parameters that contribute to the hydrolysis of plastics in the marine environment (Le Digabel and Avérous, 2006).

The hydrolytic degradation of polymers takes place in the presence of water containing an acid or a base as a catalyst (Venkatachalam et al., 2012). In an acidic environment, the main mechanism of polymer hydrolysis is different from that operating in an alkaline environment (Figure 1.3.5 a, b) (Xia et al., 2014). However, the product of both of these reactions creates one carboxyl and one hydroxyl end-group. Therefore, the reaction can be followed by measuring the increase in the number of carboxyl ends with time (Al-AbdulRazzak and Jabarin, 2002).

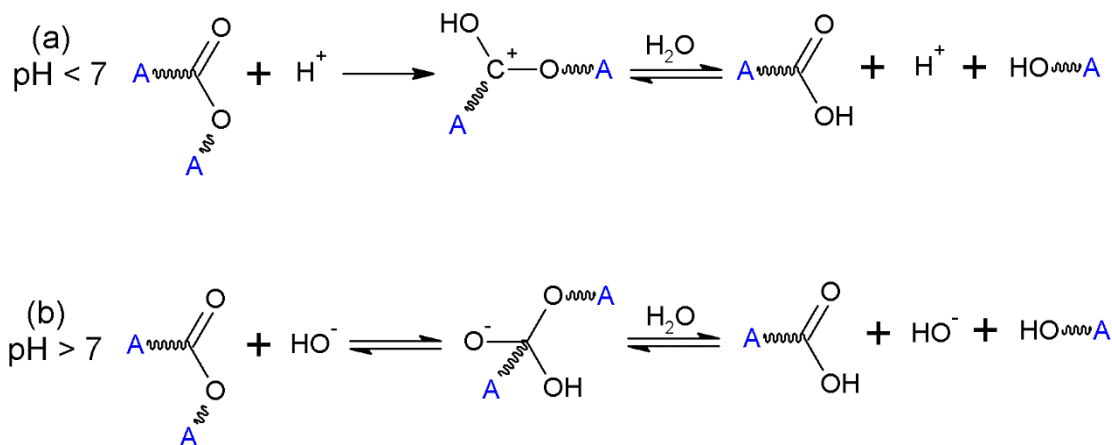


Figure 1.3.5 The hydrolytic cleavage of an ester bond under (a) Acidic and (b) Alkaline conditions.

The hydrolysis of semi-crystalline polymers such as anhydrides, amides and esters occur usually in two stages. In the first stage, water diffusion occurs in the amorphous region of plastics, which results in the random hydrolytic cleavage of the polymer and subsequent changes in morphological and mechanical properties. In the second stage, water penetrates the crystalline region and eventually degrades the crystalline region (Kint and Muñoz-Guerra, 1999). This stage involves measurable weight loss in addition to further chain cleavage. Moreover, the erosion rate usually increases over time after the onset of the second stage due to decreased crystallinity and molecular weight, as well as increased water solubility which turns the polymer into a sponge-like porous body (Pirzadeh et al., 2007).

The hydrophilic and hydrophobic nature of polymeric materials influences their degradation rate, and the susceptibility to hydrolysis follows this order: (1) hydrophilic polymers with hydrolysable bonds (e.g. PVOH, cellulose), (2) hydrophobic polymers with hydrolysable bonds (PET, PU), and (4) hydrophobic with no hydrolysable bonds (PE, PVC, PP) (Williams and Zhong, 1994, Azevedo and Reis, 2005, Min et al., 2020). There are also several factors that affect hydrolytic cleavage of the plastics which include pH, temperature, degree of crystallinity, morphology and porosity of the material. These factors influence the water permeability and therefore the erosion rates. Additionally, enzymes (e.g. lipase, protease, esterase) also play vital role in the hydrolytic degradation of plastics as they act as catalysts in these reactions (Shah et al., 2008, Donelli et al., 2009, Sangale et al., 2012, Tribedi et al., 2012, Hung et al., 2016, Wilkes and Aristilde, 2017). Such enzymatic hydrolysis of plastics is influenced by various physicochemical properties including molecular weight, degree of crystallinity, surface area, crosslinking, porosity and chemical composition of the plastics. Moreover, inherent properties of the enzymes such as solubility, activity and 3-D conformation along with extrinsic factors including pH, oxygen, temperature, and nutrient

availability also affect the enzyme catalysed hydrolytic degradation of plastics (Azevedo and Reis, 2005).

#### **1.3.3.4 Mechanical Degradation**

Mechanical shearing is an important degradation process of plastics within marine environments. It involves the degradation of plastics under mechanical stress, compression and/or tension and usually occurs due to combined actions of waves and tides (Corcoran et al., 2009). Sediment particles can also scratch the surface of plastics which causes an increase in the rate of fragmentation (Fotopoulou and Karapanagioti, 2012). The weathering of plastic by various environmental factors often changes their properties which leads to embrittlement (Duwez and Nysten, 2001). When brittle, plastic is then fragmented into microplastics due to the mechanical forces exerted during the movement through various marine habitats (Kooi et al., 2018). This mechanical degradation can further shred the microplastics into nano-plastics (size 1-1000 nm) (Lambert and Wagner, 2016, Gigault et al., 2018). However, in both cases, mechanical degradation decreases the polymer particle size and thus increases the surface area and polarity of the plastic fragments (Klein et al., 2018). These surface alterations can also lead to faster degradation of plastic particles due to higher reactivity and facilitate the adsorption of persistent organic pollutants (POPs) to the plastics (Fotopoulou and Karapanagioti, 2012, Fotopoulou and Karapanagioti, 2015).

Mechanical degradation of plastic debris may occur offshore, onshore or when plastic is deposited on a beach. At sea, there may be collisions in areas of high shipping traffic and in areas where debris density is high (Cooper and Corcoran, 2010). In addition, the mechanical degradation of plastic debris is higher on a beach than at sea because of higher mechanical forces presented by the former (Corcoran et al., 2009). The plastic debris samples collected from the beaches of Kauai, Hawaii, were observed to have crack, notches, pits, flakes and grooves on their surfaces. These mechanically produced surface textures provide the sites for oxidative processes to occur, which further weakens polymer surfaces leading to embrittlement. Fourier transform infrared spectroscopy (FTIR) results suggest that polyethylene is more liable to such oxidation compared to polypropylene and it occurs along the cracks and in the pits, created during collisions (Cooper and Corcoran, 2010).

#### **1.3.3.5 Biodegradation**

Biodegradation of plastics is a mineralisation process which results in the partial or total conversion of plastics into biogas and biomass by the activity of microorganisms (e.g.

bacteria, fungi) (Shah et al., 2008). Depending on the microbes and the respiratory conditions, the end product of the biodegradation process is either carbon dioxide (CO<sub>2</sub>) and water (H<sub>2</sub>O) under aerobic conditions, or CO<sub>2</sub> and methane (CH<sub>4</sub>) under anaerobic conditions (Mohan, 2011). Generally, biodegradation is considered to occur after or with physical and chemical degradation (abiotic degradation), which weakens the structure of polymers as revealed by morphological and molecular changes (Reisser et al., 2014). The alteration of plastic properties due to abiotic degradation is called “ageing” and depends on several factors such as temperature, sunlight and chemicals that enhance the degradation rate by oxidizing or disrupting the length of the polymer chain. Abiotic degradation only lowers the molecular weight of the material by breaking bonds and increasing the surface area whereas biodegradation, involving microorganisms and their enzymes, results in the complete degradation of plastics into CO<sub>2</sub> and H<sub>2</sub>O (Shah et al., 2008).

There are four steps that summarise the biodegradation process, which have been described in detail in a review by Dussud and Ghiglione (2014):

- Bio-deterioration – This is the first step of the biodegradation process which involves the development of a biofilm on the surface and inside the plastic. The biofilm formation increases the pore size and provokes cracks that weaken the physical properties of the plastic (physical deterioration). It also releases acidic compounds which modify the pH inside the pores and results in changes in the molecular structure of plastics (chemical deterioration) (Figure 1.3.6).
- Bio-fragmentation – A process where extracellular enzymes (e.g. esterases, lipases, peroxidases and depolymerases) are released by bacteria colonizing a polymer surface. These enzymes reduce the molecular weight by breaking the plastic into smaller units, oligomers, and then into monomers that are assimilated by microorganisms.
- Assimilation – Oligomers of less than 600 Daltons are transported inside microbial cells to be used as a carbon source. This increases the microbial biomass and produces various metabolites.
- Mineralization – The ultimate step of plastic biodegradation which results in the excretion of simple (e.g. CH<sub>4</sub>) and completely oxidized metabolites (e.g. CO<sub>2</sub>, H<sub>2</sub>O) to the extracellular surroundings (Figure 1.3.6).

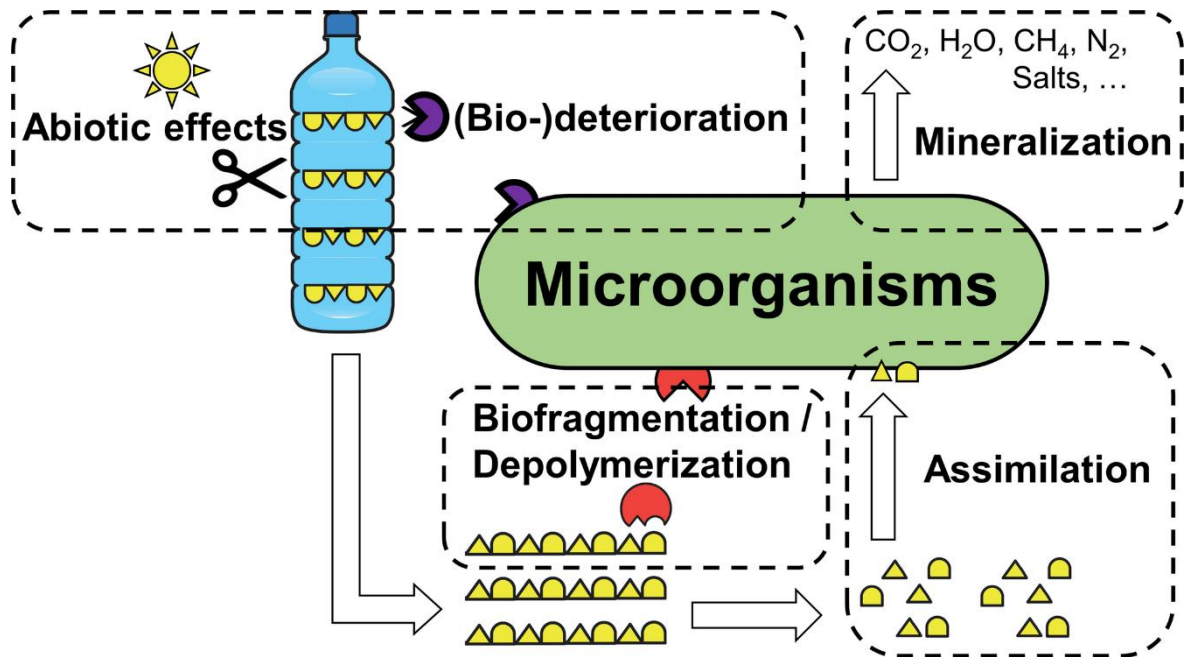


Figure 1.3.6 Various steps of plastic biodegradation in the marine environment. Original diagram by (Wei and Zimmermann, 2017)

## 1.4 Interactions Between Marine Microorganisms and Plastics

### 1.4.1 Potential Impacts of Plastics on Marine Microorganisms

Microorganisms dominate every corner of the biosphere, especially in the oceans where a single drop of seawater contains approximately 2 million microbes (Amaral-Zettler et al., 2010). Marine microbes, including auto- and heterotrophic bacteria, marine fungi, archaea, and single-celled eukaryotes called protists, are metabolically versatile and account for approximately 90 % of the total biomass of the oceans (Fuhrman et al., 1989, Whitman et al., 1998, Kirchman, 2008). They feed on carbon sources including detritus, phytoplankton exudates, and organic material released by protozoan or zooplankton grazing and carry out half of the oceanic primary productivity (Legendre and Le Fèvre, 1995, Amaral-Zettler et al., 2010). They also play a key role in maintaining biogeochemical cycles and functioning of the marine food web by decomposing and assimilating the marine dissolved organic matter (DOM) and particulate organic matter (POM) (Falkowski et al., 2008, Pomeroy et al., 2007, Kujawinski, 2011). Assimilated DOM/POM is returned to higher trophic levels through a microbial loop, a depiction of the microbial food web can be seen in Figure 1.4.1 (Azam et al., 1983, Pomeroy et al., 2007). The microbial loop is complex due to many interactions between organisms and the involvement of marine viruses, which can prevent the POM from migrating up trophic levels via a viral shunt pathway (Weinbauer et al., 2007).



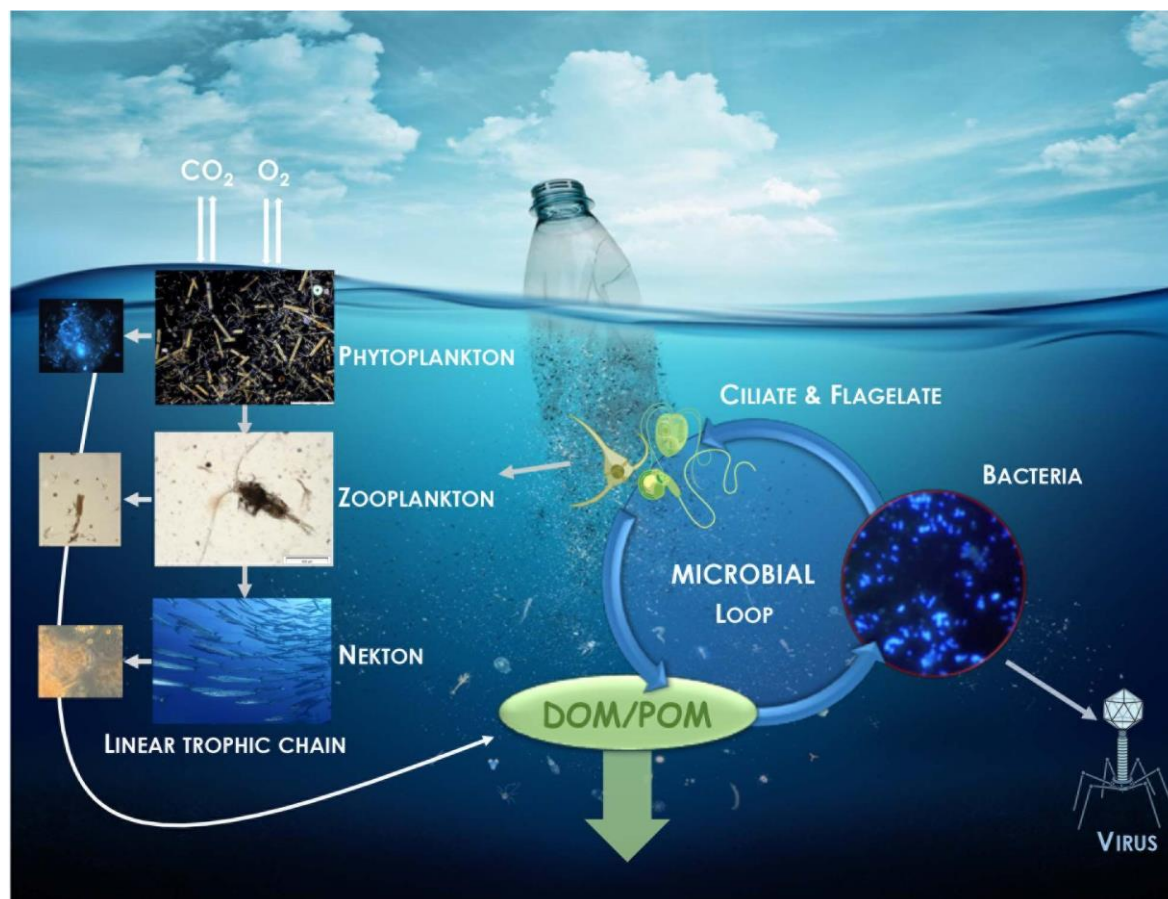


Figure 1.4.1 Overview of the potential impact of plastic on the regulation of carbon and other nutrient cycles. Dissolve (DOM) and particulate (POM) organic matter originated from the linear trophic chain is returned to higher trophic levels via its incorporation in bacterial biomass. Original diagram by Jacquin et al. (2019).

The ecological impacts of marine plastic debris are well documented for marine fauna ranging from small crustaceans to large marine animals (Andrady, 2011, Gall and Thompson, 2015) (see Section 1.1.1). However, the potential impacts of plastics on marine microorganisms are largely unknown. Only one recent study relating to plastic leachate effects on marine bacteria reported short-term growth stimulation of marine heterotrophic bacterioplankton and suggested potential consequences for marine microbial activity and carbon cycling in the oceans (Romera-Castillo et al., 2018). However, this study did not address the effects on marine primary producers. In contrast, Dussud et al. (2018) compared the heterotrophic production of bacteria living on plastic and in seawater. This study reported that heterotrophic bacteria living on plastics were particularly active, with a cell-specific activity of 43- to 88-fold higher than that of the free-living fraction. Dussud et al. (2018) focused on the microbial colonisation of plastic surfaces and therefore did not further explore the potential consequences of plastics on marine microbial ecology.

Determining the impacts of plastics on marine microorganisms that support the functioning of the marine ecosystems represent a significant research gap (Harrison et al., 2011). Apart from controlling the marine ecosystem functioning, marine microbes also serve as sensitive

indicators of ecosystem health because of their ability to modulate the metabolism in response to environmental changes (Chiu et al., 2008, Mojtahid et al., 2008, Harrison et al., 2011). In this respect, it is pertinent to study the interaction of marine microbes with plastic pollution, which may have potential consequences on marine microbial communities and microbially mediated biogeochemical processes (Hutchins and Fu, 2017).

#### **1.4.2 Microbial Attachment and Biofilm Formation on Marine Plastics**

Once in the marine environment microbes can readily colonise plastic debris (Zettler et al., 2013). As with any hard surface in the marine environment, plastics offer an ideal environment for colonisation of marine biota, which includes bacteria, fungi, diatoms, unicellular eukaryotes, and invertebrate species (Zettler et al., 2013, Harrison et al., 2014, Oberbeckmann et al., 2014, Reisser et al., 2014, Bryant et al., 2016, De Tender et al., 2017a, Kettner et al., 2019). The presence of microorganisms on marine plastic was first reported in 1972 when diatoms and rod-shaped Gram-negative bacteria were observed on plastic fragments collected from the Sargasso Sea (Carpenter and Smith, 1972, Carpenter et al., 1972). Similarly, a few years later, microbial colonisation was also identified on various anthropogenic materials, which included high-density polyethylene plastic (HDPE) bottles using Scanning Electron Microscopy (SEM) (Sieburth, 1975). After these initial observations of diatoms and bacterial colonisations, the topic did not receive much attention until recently (Harrison et al., 2011), with Zettler et al. (2013) reporting the first comprehensive characterisation of microbial communities on marine plastic debris using a culture-independent and next-generation sequencing (NGS) approach. The study showed that physically and chemically distinct man-made plastic materials supported and selected for distinct microbial communities which were referred to as the “Plastisphere”. These “Plastisphere” communities were also different from surrounding seawater communities (Zettler et al., 2013). The term “Plastisphere” was initially assigned to the assemblage of taxa inhabiting on the surface of marine plastic debris but is now generally referred to the biota living on the surface of the plastic debris (De Tender et al., 2017a)

The colonisation of microbial communities on plastic surfaces occurs through the formation of a biofilm. The marine microorganisms undergo remarkable changes during the biofilm formation, i.e. from planktonic (free-living) to complex, surface-attached communities (Figure 1.4.2). The formation of a biofilm generally starts with the preconditioning of the polymer with inorganic and organic substances which are adsorbed immediately onto the surface after first contact with seawater (Loeb and Neihof, 1975). Once the microorganisms

attach to a polymer surface they then start to colonise. The initial attachment is reversible, however at this point microorganisms will begin to form microcolonies and produce an extracellular matrix. This matrix consists of extracellular polysaccharides, cell debris, structural proteins and nucleic acids and is usually referred to as extracellular polymeric substances (EPS) (Flemming and Wingender, 2010). The EPS act as an adhesive and supports the irreversible attachment of microcolonies. The biofilm further grows through cell-to-cell communications (quorum sensing) which play a key role in the biofilm maturation (Waters and Bassler, 2005). At the end of the biofilm growth cycle, microbial cells begin to disperse as plankton cells start a new cycle of biofilm formation (Rendueles and Ghigo, 2012) (Figure 1.4.2).

Formation of a microbial biofilm on marine plastic materials occurs very quickly (e.g. after one week) (Lobelle and Cunliffe, 2011). Similarly, bacterial colonisation of low-density polyethylene (LDPE) occurred after 7 days of exposure in marine sediments (Harrison et al., 2011). In both cases the biofilms were mainly dominated by bacteria and diatoms, along with marine invertebrates (Carson et al., 2013, Reisser et al., 2014). To date, most of the plastic biofilm studies have focused on comparing the colonising communities to that of seawater and have reported striking differences between the “Plastisphere” and surrounding seawater communities (Zettler et al., 2013, Amaral-Zettler et al., 2015, De Tender et al., 2015, Bryant et al., 2016). From the composition perspective, spatial and seasonal effects (Amaral-Zettler et al., 2015, Oberbeckmann et al., 2016), geographical location (De Tender et al., 2017a), substrate type (Oberbeckmann et al., 2014, Martin et al., 2018) and polymer surface properties (e.g. roughness, free energy and hydrophobicity) (Carson et al., 2013, Rummel et al., 2017) are known to be the controlling parameters for the development of distinct marine plastic biofilms. It is, however, well established that marine plastic biofilms contain various general bacterial families including primary colonisers of family *Rhodobacteraceae*, *Hyphomonadaceae*, *Erythrobacteraceae*, and *Pelagibacteraceae* (Dang et al., 2008, Zettler et al., 2013, Bryant et al., 2016, Oberbeckmann et al., 2018). Members of *Flavobacteriaceae*, *Cytophagaceae* and *Sphingobacteriaceae* appear as secondary colonizers (Dang and Lovell, 2000, Lee et al., 2008) on mature marine plastic biofilms (Bryant et al., 2016, De Tender et al., 2017a).

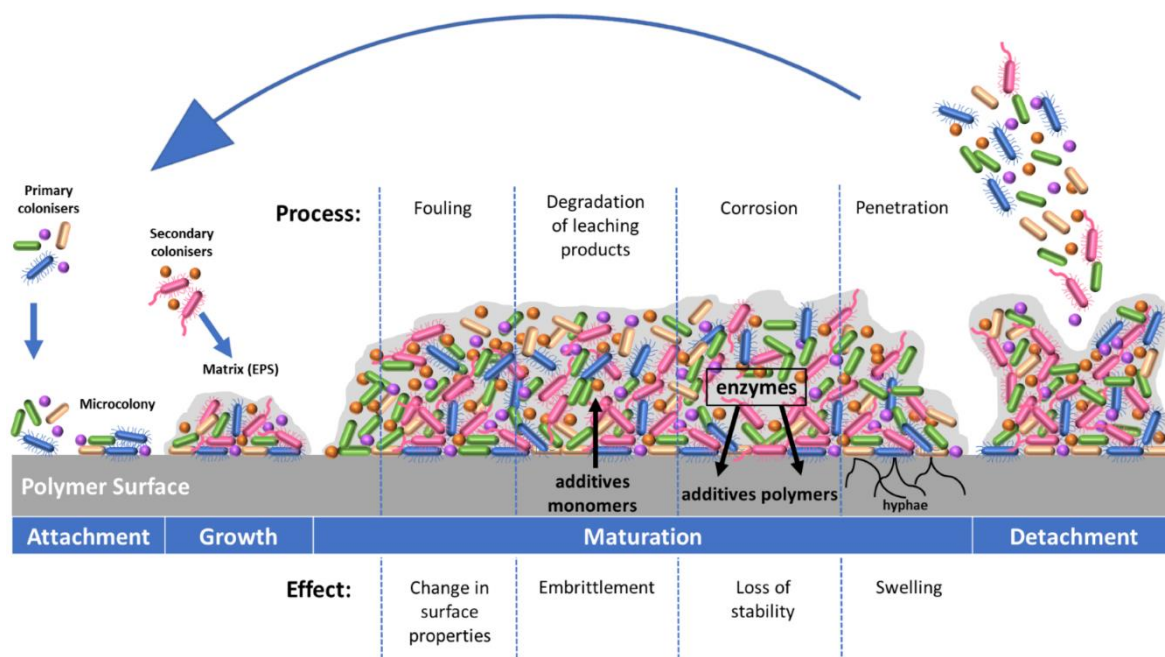


Figure 1.4.2 Stages of biofilm formation and development. Redrawn and adapted from (Flemming, 1998, Rendueles and Ghigo, 2012)

Despite the fact that microorganisms can colonise all plastics that are introduced into the marine environment (De Tender et al., 2015, Eich et al., 2015, Rummel et al., 2017), the investigations of biofilm formation on plastic surfaces are scarce (De Tender et al., 2017a). Only a few studies have so far described either the early (< 4 weeks) (Lobelle and Cunliffe, 2011, Harrison et al., 2014, Eich et al., 2015, Oberbeckmann et al., 2016) or the long term (> 6 months) biofilm formation (Webb et al., 2009, De Tender et al., 2017a) onto a limited number of plastic materials. These studies were focused on either one plastic-type or they compared it with the communities of other non-plastic substrates e.g. wood and glass. Some studies have however analysed the biofilm communities on randomly collected plastic debris (Oberbeckmann et al., 2014, Bryant et al., 2016, Debroas et al., 2017). The biofilm development process on these randomly sampled plastics is not clear due to their unknown source, exposure period and travel history (De Tender et al., 2017a). Consequently, there is a lack of ecological information about the specificity and temporal dynamics of biofilm formation on chemically distinct synthetic plastics such as polyesters and polyolefins under comparable marine environments. Addressing this research gap forms one of the aims of this thesis.

### 1.4.3 Plastic Degradation by Marine Microbes

Plastics are potential substrates for heterotrophic marine microorganisms including bacteria and fungi. The biodegradability of plastics depends on the physical state, crystallinity, molecular weight and the metabolic interactions within plastic-attached biofilms (Gu, 2003,

Artham et al., 2009b, Harrison et al., 2011). Generally, the biodegradation of plastics within marine environments involves various steps during which plastics are cleaved into smaller units (e.g. oligomers and monomers) that can further be assimilated by microbes (Lucas et al., 2008, Dussud and Ghiglione, 2014) (Section 1.3.3.5, Figure 1.3.6). Moreover, the formation of biofilms on a plastic surface is very crucial in their biodegradation (Flemming, 1998, Orr et al., 2004b), which can result in different mechanisms of polymer degradation as shown in Figure 1.4.2 (Section 1.4.2). However, the durability of recalcitrant plastic materials and their lower bioavailability to microbes are the main obstacles to biodegradation in the marine environment (Lucas et al., 2008, Gewert et al., 2015).

The ability of microorganisms to degrade plastics have been known for several decades, with the first study in 1968 identifying bacterial isolates with the potential to degrade plastic (Booth et al., 1968). However, the biodegradation process of plastics within the marine environment became a focus of research a couple of decades later (Palmisano and Pettigrew, 1992). So far, there are only a few studies that have analysed the biodegradation potential of a wide range of plastics in the marine environment (Table 1.4.1). These studies are mostly based on the isolation and *in vitro* biodegradability testing of microbial strains isolated from marine environments. The isolated microorganisms, including marine bacteria and fungi, showed the biodegradation potential of plastics (Urbanek et al., 2018) which was assessed based on weight loss. For example, Balasubramanian et al. (2010) isolated bacterial strains belonging to genera *Pseudomonas* and *Arthrobacter* from the Gulf of Mannar, India and measured a 15 % decrease in HDPE weight. Similarly, bacteria genera such as *Bacillus* and *Rhodococcus* were reported to reduce the weight of polyethylene by 7.4 % (Auta et al., 2017, Auta et al., 2018). In contrast, some marine fungi have also been identified as plastic degraders, e.g. *Aspergillus* sp. which degraded HDPE by 8.5 % (weight loss) after a 30 day incubation (Devi et al., 2015) and *Zalerion maritimum* which caused microplastics of polyethylene to lose 43% of its weight after 14 days (Paço et al., 2017). The other microbial taxa isolated from marine environments with the ability to utilise plastic materials as a source of carbon and energy are listed in Table 1.4.1. Whilst marine microbes have demonstrated the capabilities to degrade plastics, the rate of biodegradation is slow, even in optimised laboratory conditions (Krueger et al., 2015, Debroas et al., 2017). This has been attributed to the large size of plastic molecules, higher chemical stability and lower bioavailability of plastic materials to microbial cells (Alexander, 1975, Andrady, 1994). Additionally, low levels of oxygen, light and nutrients within the marine environment may also limit the plastic biodegradation process (Barnes et al., 2009, Jacquin et al., 2019).

Although existing studies provide the evidence for microbial degradation of plastics in marine environments (Table 1.4.1), little is known about the complete assimilation and mineralisation of plastics by marine microbes. Recently, a bacterial strain, *Ideonella sakaiensis*, was isolated from sediment of a plastic recycling facility in Japan, which demonstrated the complete assimilation and mineralisation of PET using two enzymes (Yoshida et al., 2016). This indicated that high concentration of plastics can induce evolution in bacteria for plastic biodegradation. With the increasing amounts of plastic debris in the oceans this might also be true for marine bacteria. Moreover, the colonisation of potential biodegraders on plastics has also been observed in marine habitats (Zettler et al., 2013, Harrison et al., 2014) and recently Bryant et al. (2016) reported the presence of xenobiotic biodegradation genes in relation to the attached microbes on marine plastic surfaces. The processes underlying the attachment of these biodegraders onto marine plastics and their ability to degrade are largely unknown (Harrison et al., 2018b), but these findings imply that biodegradation potential of marine microorganisms is ubiquitously present in the world's oceans. Taking into consideration that most marine microorganisms potentially participating in the degradation of plastic debris remain undiscovered, this thesis aimed, in part, to isolate and characterise indigenous marine species with the potential to degrade plastics.

Table 1.4.1 List of microorganisms isolated from the marine environment with the capability of degrading various plastics

Microorganisms	Sources	Plastics	References
<b>Bacteria</b>			
<i>Bacillus sphaericus</i> and <i>Bacillus cereus</i>	Indian Ocean	LDPE, HDPE and starch-blended LDPE	(Sudhakar et al., 2008)
<i>Pseudomonas</i> sp.	Deep seawater	Polycaprolactone (PCL)	(Sekiguchi et al., 2009)
<i>Arthrobacter</i> sp. And <i>Pseudomonas</i> sp.	Gulf of Mannar, India	HDPE	(Balasubramanian et al., 2010)
<i>Alcanivorax</i> sp., <i>Pseudomonas</i> sp. and <i>Tenacibaculum</i> sp.	Deep seawater	PCL, PHB/V and Polybutylene succinate (PBS)	(Sekiguchi et al., 2011a)
<i>Shewanella</i> sp., <i>Moritella</i> sp., <i>Psychrobacter</i> sp. and <i>Pseudomonas</i> sp.	Deep-sea sediments	PCL	(Sekiguchi et al., 2011b)
<i>Kocuria palustris</i> M16, <i>Bacillus pumilus</i> M27 and <i>Bacillus subtilis</i> H1584	Pelagic zone, Arabian Sea, India	LDPE	(Harshvardhan and Jha, 2013)
<i>Vibrio alginolyticus</i> and <i>Vibrio parahaemolyticus</i>	Benthic zones of marine environments	Polyvinyl alcohol (PVA) blended low LDPE	(Raghul et al., 2014)
<i>Lysinibacillus</i> sp. and <i>Salinibacterium</i> sp.	Plastic samples from the North Sea	Weathered LDPE	(Syranidou et al., 2017)
<i>Pseudomonas</i> sp. and <i>Rhodococcus</i> sp.	Arctic soil	PCL, potato and corn starch based commercial bag	(Urbanek et al., 2017)
<i>Bacillus gottheilii</i> and <i>Bacillus cereus</i>	Mangrove ecosystems in Peninsular Malaysia	PE, Polypropylene (PP), PS	(Auta et al., 2017)
<i>Brevibacillus borstelensis</i>	Marine sediment	HDPE	(Mohanrasu et al., 2018)
<i>Bacillus</i> sp. strain 27 and <i>Rhodococcus</i> sp. strain 36	Mangrove ecosystems in Peninsular Malaysia	PP	(Auta et al., 2018)
<i>Bacillus flexus</i> and <i>Pseudomonas citronellolis</i>	Marine environment	PVC	(Giacomucci et al., 2019)
<i>Bacillus</i> sp. and <i>Pseudomonas</i> sp.	Coast of Tamil Nadu, India	HDPE	(Sangeetha Devi et al., 2019)
<b>Fungi</b>			
<i>Aspergillus versicolor</i> , and <i>Aspergillus</i> sp.	Bay of Bengal, India	LDPE	(Pramila and Ramesh, 2011)
<i>Aspergillus tubingensis</i> VRKPT1 and <i>Aspergillus flavus</i> VRKPT2.	Gulf of Mannar, India	HDPE	(Devi et al., 2015)
<i>Trichoderma</i> sp. and <i>Clonostachys Rosea</i> ,	Arctic soil	PCL, potato and corn starch based commercial bag	(Urbanek et al., 2017)
<i>Zalerion maritimum</i>	Marine environment	PE	(Paço et al., 2017)

# **Chapter 2: A Time-Series Investigation into the Effects of Plastic Pollution on Microbial Community Structure and Diversity in Seawater: A Microcosm Study**

## **2.1 Introduction**

Marine microbial communities, encompassing single-cell bacteria, fungi, archaea and eukaryotes, dominate the World's oceans due to their overwhelming abundance and metabolic versatility within marine ecosystems (Kirchman, 2008, Amaral-Zettler et al., 2010). They carry out approximately half of the primary production and play an important part in nutrient cycling (Arrigo, 2004). More than 95 % of respiration in the oceans is attributed to microbial activity (Del Giorgio and Duarte, 2002). Marine microbes are also the driving force behind biogeochemical cycling including iron, sulphur and manganese, and govern nitrogen utilisation through nitrogen fixation (Kirchman, 2008). Moreover, heterotrophic marine bacteria support the lower level of marine food webs by decomposing organic carbon and re-mineralising inorganic nutrients (Pomeroy et al., 2007). The assimilated carbon and energy from bacteria are transferred to larger zooplankton through protozoan grazing. The resulting microbial loop creates a link between marine bacteria and higher trophic levels (Azam et al., 1983, Pomeroy et al., 2007).

Human activities resulting from dumping or incidental release of pollutants, coastal engineering, fishing, tourism, maritime transport and mining are constantly exerting pressure on the marine ecosystem (Nogales et al., 2011). Despite their enormous functional resilience, the prokaryotic and eukaryotic marine microorganisms are also facing these unprecedented challenges (Hutchins and Fu, 2017). These anthropogenic perturbations are altering almost all physical, chemical and biological properties in the ocean which could affect the growth of marine microorganisms. Nevertheless, it is unclear how these anthropogenic activities will reshape the abundance and diversity of marine microbial communities, which form the base of the ocean's life support system (Hutchins and Fu, 2017).

One of the most direct human impacts on present-day oceans is pollution from plastic waste. It is ubiquitous within marine ecosystems and has become a global concern due to its



detrimental effects on aquatic wildlife (Thompson et al., 2009). The majority of the waste enters the world's oceans from terrestrial sources (Wright et al., 2013b) and accumulates in gyres due to ocean circulation patterns (Eriksen et al., 2013, Cózar et al., 2014). It is estimated that 275 million metric tons (MMT) of plastic waste was generated in 2010, with 4.8 to 12.7 MMT entering the ocean. By 2025, it is predicted that an increase by one order of magnitude is predicted for the cumulative quantity of plastic waste entering the oceans from land (Jambeck et al., 2015), with potentially large implications for marine life.

As comparatively new to the marine environment, plastic debris serves as a substrate for microbes, which lasts longer than other naturally occurring substrates in the marine ecosystem (Zettler et al., 2013). The surface of plastics provides a suitable environment for microbial colonization, with several marine physicochemical factors, e.g. climate, light, and nutrient loading, influencing the microbial community succession (De Tender et al., 2017b, Bunse and Pinhassi, 2017). Microbial colonisation of the plastics occurs relatively fast (Lobelle and Cunliffe, 2011, Harrison et al., 2014) similar to other artificial surfaces (glass, steel and acryl) (Salta et al., 2013) and colonising communities are taxonomically distinct from those in the surrounding water column (Zettler et al., 2013). However, little is known about the nature of plastic-microbe interactions and the effect plastic has on marine microbial communities. More importantly, it is also needed to study the effects of substrate type (synthetic and bio-based/degradable plastics) on marine microbial diversity as the nature of litter is likely to evolve in the future with the advent of new biodegradable plastics.

This chapter focuses on the changes in the diversity and composition of marine bacterial communities caused by the addition of various chemically distinct natural and synthetic plastics. The effects of plastics on marine microbes could be subtle and observed only after the resultant changes have become noticeable through higher trophic levels. Therefore, the characterisation of microbial communities within seawater containing plastics is paramount as it would allow to identification of the factors contributing to the impacts on the lower level of marine food webs.

The two aims of this chapter are:

- (1) to assess the variation in the community structure and diversity of marine bacterial community due to the presence of various plastics over time; and
- (2) to identify the main physio-chemical properties of plastics and seawater driving the resultant variation.

## 2.2 Materials and Methods

### 2.2.1 Seawater Sampling

A stimulated marine environment was developed to investigate the effect of various plastics on diversity and structure of marine bacterial communities using freshly collected seawater. The natural seawater (NSW) samples were collected from Skegness beach (53.137844 °N; 0.351808 °E), the UK on 20 April 2017 (Figure 2.2.1). Samples were obtained prior to high tide from the top 1 m water column in 10 L capacity containers for transit. Samples were stored either overnight in darkness at 4 °C for use in laboratory microcosms, or at -20 °C for DNA extraction. The water was pre-filtered using Whatman<sup>®</sup> quantitative filter paper, Grade 40 (pore size 8 µm) to remove large organisms that may feed on bacteria during the experiment. In addition, temperature (12.8 °C), salinity (38.2) and dissolved oxygen (9.36 mg/L) was determined at the time of sampling, using a 395 handheld refractometer (Test Products Intl. Ltd, Sussex, UK ) and portable HI98193 DO meter (HANNA instruments, UK) respectively. The pH of the samples ( $7.5 \pm 1$ ) was also measured upon arrival to the lab using Thermo Scientific<sup>™</sup> Orion<sup>™</sup> Versa Star Pro<sup>™</sup> (Fisher Scientific, Loughborough, UK).

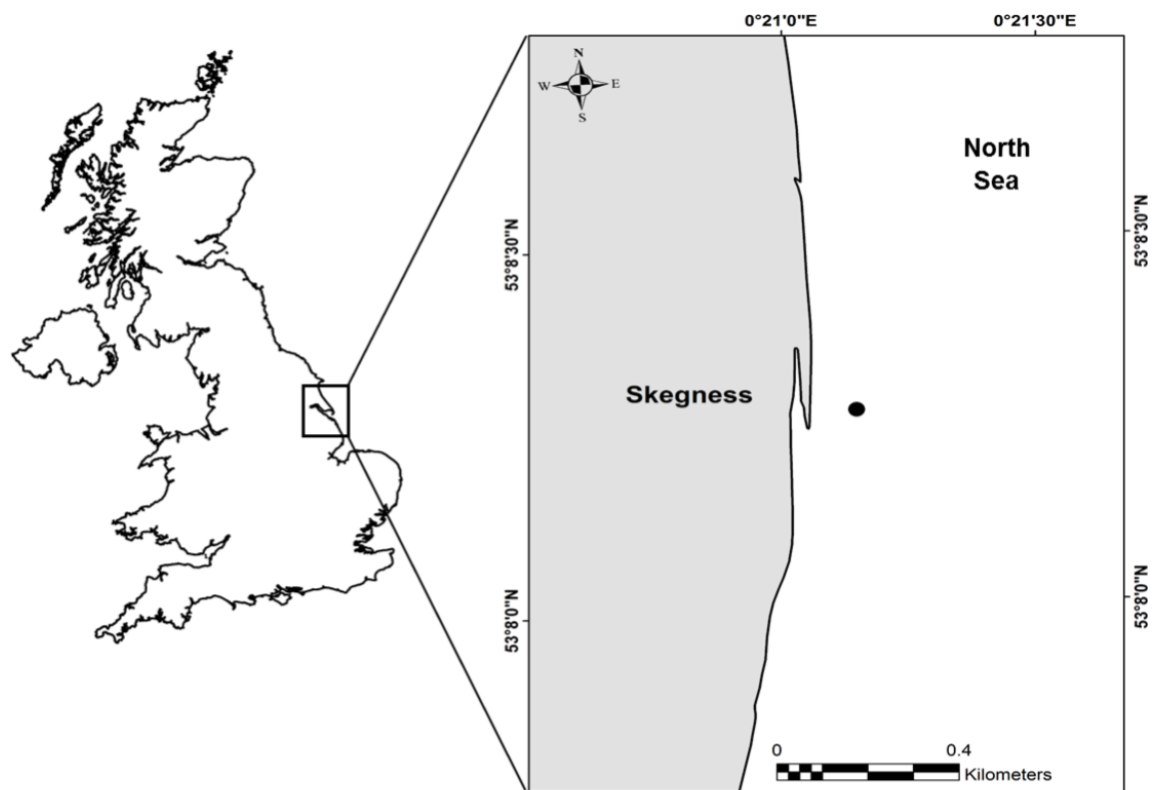


Figure 2.2.1 Location of the seawater sampling site. The small black box denotes the regional location of the sampling sites within the UK. Location of field site used for seawater sampling at Skegness, Lincolnshire, UK is shown in the magnified box. Circular marker denotes the sampling site.

## 2.2.2 Seawater-Plastics Microcosms Setup

Seven different plastic materials (referred to as treatments) were selected ranging from environmentally persistent fossil fuel-derived synthetic polymers to naturally produced biodegradables (Table 2.2.1). Each plastic along with the freshly collected NSW was added to a 500 mL autoclaved Erlenmeyer flask to create a microcosm. Eight sets of microcosms, one for each test plastic and for the control (with no plastic) were set up in triplicate. Each microcosm contained 300 mL of NSW spiked with 100 mg of plastics (sterilised with 70 % ethanol). The flasks were incubated in a Microclima 1750 climate cabinet (Snijders Labs, Tilburg, The Netherlands) at 15 °C, 80 % relative humidity, in the dark at 400 ppm CO<sub>2</sub> atmospheric concentration for 512 hrs. These conditions were based on *in situ* measurements of the NSW sample site and representative of marine environments. The flasks were also rotated at the speed of 50 rpm to facilitate aeration during the experiment. A sacrificial sampling of 20 mL seawater from all microcosms was performed aseptically in triplicate (totalling 24 per time point) at 10 intervals (1, 2, 4, 8, 16, 32, 64, 128, 256 and 512 hrs). The samples were filtered through Whatman<sup>®</sup> quantitative filter paper, Grade 42 (pore size 2.5 µm) to remove plastic fragments from the water. Sub-samples of seawater after filtration was also withdrawn for molecular analysis at each time intervals and stored at -80 °C.

Table 2.2.1 Name and properties of the plastics used in the Study

Polymer type*	Abbreviation	Particle size	Form	Colour	Manufacturer
<b>Synthetic (fossil-based, non-biodegradable)</b>					
Polyethylene terephthalate	PET	3 mm	Pellet	White	Goodfellow Cambridge, UK
High-density Polyethylene	HDPE	2-4 mm	Pellet	Natural	Goodfellow Cambridge, UK
Low-density Polyethylene	LDPE	1 mm	Pellet	Natural	Goodfellow Cambridge, UK
Polyvinyl chloride	PVC	0.25 mm	Pellet	Light Gray	Goodfellow Cambridge, UK
<b>Biodegradable (fossil-based)</b>					
Poly (vinyl alcohol)	PVOH	Refined particles	Powder	White	Sigma Aldrich, Germany
<b>Biodegradable (bio-based)</b>					
Polylactic acid	PLA	3 mm	Pellet	White	Goodfellow Cambridge, UK
Polyhydroxybutyrate /Polyhydroxyvalerate	PHB/PHV	5 mm	Pellet	White	Goodfellow Cambridge, UK

\*All plastics were of research grade and contains no additives or stabilisers.

### **2.2.3 Physico-Chemical Characterisation of Seawater**

The physicochemical parameters of the seawater were characterised during the experiment using the following methods:

- pH measurement;
- Dissolved inorganic carbon (DIC), dissolved organic carbon (DOC) and total nitrogen (TN) determination.

#### **2.2.3.1 Seawater pH Measurements**

Variation in pH is indicative of microbial growth due to the production of various metabolites as by-products of microbial metabolism (Sumbali and Mehrotra, 2009). The pH of NSW (control and plastic-treated) was monitored aseptically throughout the experiment using a Thermo Scientific™ Orion™ Versa Star Pro™ (Fisher Scientific, Loughborough, UK) with a three-point calibration using Omega Buffer solutions at pH 4, 7 and 10.

#### **2.2.3.2 Dissolved Inorganic Carbon (DIC), Dissolved Organic Carbon (DOC) and Total Nitrogen (TN) Determination**

The dissolved inorganic and organic carbon content of NSW (control and plastic treatment) were analysed using a Shimadzu Total Carbon analyser (TOC-V<sub>CSN</sub>) instrument. DIC was measured by acidifying 10 ml of sample with 2 M hydrochloric acid (HCl). The addition of acid converted all the carbonates and dissolved CO<sub>2</sub> in the sample to gaseous carbon dioxide, which was subsequently measured by an infra-red detector. Dissolved organic carbon analysis used a non-purgeable organic carbon (NPOC) method. After eliminating the IC component by acidifying and purging with CO<sub>2</sub> free gas, the remaining sample was injected into a combustion tube (680 °C) containing a platinum catalyst. The resulting CO<sub>2</sub> was measured by an infra-red detector.

The instrument was calibrated using various concentrations (0-100 mg L<sup>-1</sup>) of pre-dried reagent grade sodium hydrogen carbonate (NaHCO<sub>3</sub>) and sodium carbonate (Na<sub>2</sub>CO<sub>3</sub>) for DIC analysis. Potassium hydrogen phthalate certified standards (Sigma-Aldrich®) were used to calibrate for DOC analysis. The detection limit of the instrument for DOC analysis was below 0.1 mg L<sup>-1</sup> carbon and below 0.3 mg L<sup>-1</sup> for DIC analysis.

For total nitrogen (TN) analysis, the sample was heated at 680 °C to decompose the TN in the sample to nitrogen monoxide (NO). The resultant NO was then measured by a

Chemiluminescence gas detector. Quantitation was achieved by calibrating the TOC-V instrument with potassium nitrate (KNO<sub>3</sub>) standard.

#### **2.2.4 Microbial Community Analysis**

The seawater microbial community was characterised by using the following molecular methods:

- Cell enumerations
- Terminal restriction fragment length polymorphism (T-RFLP)
- Illumina MiSeq Barcoded Amplicon Sequencing
- *In silico* digestion of MiSeq sequences

##### **2.2.4.1 Seawater Cell Enumerations**

To determine the total cell number in the control and plastic treated NSW, 1 mL of sample was taken from each microcosm aseptically. The cells in NSW were stained with nucleic acid binding dye SYBR<sup>TM</sup> Green I DNA (Invitrogen<sup>TM</sup>, UK) to visualise under ultra-violet light. Ten µL of samples were stained with 0.2 % solution of SYBR<sup>TM</sup> Green I DNA in 1:1 ratio on a microscopic slide and incubated in the dark for 20 min at room temperature. The brightly stained cells were visualised by epifluorescence microscopy and enumerated under a Leica DM 4000 B microscope (Leica Microsystems, UK), using 485nm wavelength blue excitation filter (Marie et al., 1997). To calculate the number of cells per mL of sample, the visible cells within twenty frames of view were counted for each of the samples.

##### **2.2.4.2 Terminal Restriction Fragment Length Polymorphism (T-RFLP)**

T-RFLP is a low cost, high throughput molecular fingerprinting method, which is used to measure and compare microbial diversity within a sample. It is based on polymerase chain reaction (PCR) amplification of the target nucleic acid sequence (e.g. 16S rRNA gene) with fluorescently labelled primers (Liu et al., 1997). The resultant PCR amplicons are then cleaved using restriction endonuclease enzymes to produce terminal restriction fragments (T-RFs) of different lengths. Each T-RF can then be assigned to a relevant microbial group according to the size and the intensity of each T-RF is proportional to the abundance of the population within a sample. Following methods were used for T-RFLP analysis.

#### **2.2.4.3 Genomic DNA Extraction**

The total genomic DNA extraction was performed on the original NSW and subsamples from each of the plastic treated microcosms and controls, using the bead-beating method as described by Griffiths et al. (2000) with slight modifications. At first, 10 mL of sample was centrifuged at  $12000 \times g$  for 15 min at 4 °C to concentrate the microbial biomass. The pellet was then transferred into 2 mL Lysing Matrix E tube (MP Biomedicals, OH, USA) along with 0.5 mL of 5 % hexadecyltrimethylammonium bromide (CTAB) solution (5 g CTAB in 100 ml 120 mM  $K_2HPO_4$  buffer of pH8.0) in 120 mM phosphate buffer (pH 8.0) and 0.5 mL of phenol:chloroform:isoamyl alcohol in the ratio of 25:24:1. The tube was shaken at the speed setting of  $m.s^{-1}$  for 40 secs using an MP Fastprep-24 homogeniser (MP Biomedicals, OH, USA) and then centrifuged at  $16,000 \times g$  for 15 min at 4 °C. The aqueous phase (supernatant) was then removed and an equal volume of chloroform: isoamyl alcohol (24:1) was added to remove residual phenol, followed by centrifugation at  $10,000 \times g$  for 10 min at 4 °C. The total genomic DNA was subsequently precipitated overnight at 4 °C using  $2 \times$  volume of polyethelene glycol solution (30 % PEG 6000 in 1.6 M NaCl solution), followed by centrifugation at  $14,000 \times g$  for 15 min at 4 °C. The pelleted DNA was then washed with ice-cold ethanol (70 %), air dried and re-suspended in 50  $\mu$ L of nuclease-free water (Invitrogen <sup>TM</sup>, UK). Subsequent purification was conducted using GeneMATRIX universal DNA purification kit (EURx, Gdansk, Poland). DNA extraction yields were measured with a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE).

#### **2.2.4.4 PCR Amplification and Product Digestion**

For T-RFLP analysis, the hypervariable V1-V3 region of 16S rRNA gene within extracted genomic DNA was partially amplified using dye-labelled (6FAM) 63F (5'- CAG GCC TAA CAC ATG CAA GTC -3') (Marchesi et al., 1998) and 530R (5'- GTA TTA CCG CGG CTG CTG-3') (Muyzer et al., 1993) primers. Amplification was carried out using 1  $\mu$ L of template DNA in a 50  $\mu$ L reaction mixture containing 250 nM of each primer, 5  $\mu$ L of  $5\times$  Taq buffer, 2 mM  $MgCl_2$ , 0.1 mM of each dNTP, 5  $\mu$ g bovine serum albumin (BSA; New England Biolabs, MA) and 1.75 U of Taq (New England Biolabs, MA). PCR conditions were as follows: initial denaturation for 10 min at 94 °C followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min and a final extension at 72 °C for 10 min. All PCR products were verified on a 1.25 % (w/v) agarose gel (Sigma, Aldrich, Germany) by electrophoresis with a DNA marker, 2-Log DNA Ladder (New England Biolabs, MA).

PCR products were purified using GeneMATRIX Basic DNA Purification Kit (EURx, Gdansk, Poland) according to the manufacturer's guidelines. Purified PCR products were quantified using a Nanodrop spectrophotometer (Thermo Scientific, Wilimington, DE). 250 ng of PCR products were digested in a 25 µL reaction mixture containing 20 U *MspI* restriction endonuclease (New England Biolabs, MA), and 2.5 µL of enzyme buffer. Digestion was carried out for 4 h at 37 °C. The digested product (2 µL) was added to 9 µL of Hi-Di formamide (highly deionized formamide; Applied Biosystems, CA) and 0.35 µL of GeneScan™ 600 LIZ size standard (Applied Biosystems, CA). The mixture was incubated at 95 °C for 5 min, followed by 5 min on ice and then analysed on a 3730XL DNA sequencer (Applied Biosystems, CA) with the injection voltage of 1.6kV. Capillary electrophoresis was conducted at 15kV for 1600 sec.

#### **2.2.4.5 TRFs Data Processing**

The T-RFLP profiles were analysed using GeneMarker® software (V2.7.0 SoftGenetics, PA), using manually created bins. The terminal restriction fragments (T-RFs) presenting between 50-500 nucleotides in length and having fluorescence intensities of > 100 units were included in subsequent analysis. T-RFLP profiles were aligned using the software T-Align (<http://inismor.ucd.ie/~talign/index.html>) (Smith et al., 2005). Initially, T-align compared the replicate profiles within one sample and removed the pseudo peaks. It then generated a single consensus profile from the replicates and displayed T-RFs with average peak areas (Smith et al., 2005). To analyse the aligned T-RFLP profiles, the total peak area of each T-RF was normalised within each profile (i.e. the area under each peak was divided by the total peak area of each sample). Peaks with areas of < 0.5 % were excluded to remove the background noise of sequencer data.

#### **2.2.4.6 Illumina MiSeq Barcoded Amplicon Sequencing**

To facilitate the identification of the T-RFLP fragments, barcoded 16S rRNA gene amplicon sequencing, targeting the V1- V3 hypervariable region was used to analyse the DNA from the NSW collected for this study. Sequencing was performed using Illumina Miseq platform with the pair-end protocol at Research and Testing Laboratories (Lubbock, TX, US) using a 600 cycle (2×300 bp) v3 MiSeq reagent kit. The NSW sample was amplified with 63F-530R primers for the sequencing process. Primers also contained the Illumina sequencing adapter sequence and a unique barcode index. Resulting amplicons were paired-end sequenced which were further processed using the MiSeq SOP ([https://www.mothur.org/wiki/MiSeq\\_SOP](https://www.mothur.org/wiki/MiSeq_SOP)) cited within the MOTHUR program (Kozich et

al., 2013). Contiguous sequences were constructed from paired-end sample reads using MOTHUR (v1.39.5). All sequences, with any ambiguities or homopolymers longer than eight bases, were excluded from further analysis. All remaining sequences were aligned against a non-redundant SILVA compatible database (v.128). All sequences were trimmed to a maximum length of 478 bases before chimeric sequences were identified and removed using VSEARCH (Rognes et al., 2016). The taxonomic classification of sequences was determined by comparing with the MOTHUR formatted ribosomal database project (RDP) database (v.16). Any sequence returned as unknown, chloroplast or mitochondrial were removed from further downstream analysis. All the sequences obtained in this study are available within the NCBI Short Read Archive (SRA) database (BioProject: PRJNA454558; BioSample: SAMN09010416)

#### **2.2.4.7 *In Silico* Digestion of MiSeq Sequences**

Individual T-RF peaks can be used to differentiate between the taxonomic groups, but to provide taxonomic identification, these peaks need to be assigned a taxonomic identity. For this, *in silico* digestion of MiSeq sequences was carried out using Restriction Map ([http://www.bioinformatics.org/sms2/rest\\_map](http://www.bioinformatics.org/sms2/rest_map)). All sequences were analysed using the restriction endonuclease *MspI* cleavage site. As some T-RFs can represent various taxa, sequences which had the same cleavage site were grouped and an approximate taxon that matched at least 75 % of the sequences was assigned to that T-RF.

#### **2.2.5 Statistical Analysis**

All statistical analyses were carried out using multivariate routines in the statistical software package PRIMER 7 (v7.0.13) with PERMANOVA+ package (Clarke and Gorley, 2015). Prior to the analysis, the normalised T-RFs data were log transformed, as it down-weighted the effect of abundant T-RFs for enhancing the influence of less abundant T-RFs on the outcome of analyses, whilst still allowing the relative differences of T-RFs abundances to influence the patterns in community structure. Similarity matrices of transformed T-RFs data were constructed using the Bray-Curtis method (Clarke et al., 2006).

A visual evaluation of the similarities among treated samples was conducted using principal coordinates analyses (PCO) (Gower, 1966, Ramette, 2007). To indicate a percentage of similarity between samples on the PCO, a similarity profile test (SIMPROF) was performed on group average cluster analysis and then overlaid onto the PCO. Shannon-Weiner diversity indices of  $H'$  were generated to estimate the alpha diversity and compared using an analysis



of variance to determine significant differences between the diversity of treatments and time points sampled.

A two-factorial Permutational Multivariate Analysis of Variance (PERMANOVA) (Anderson and Walsh, 2013) analysis was conducted using transformed T-RFs abundance counts to test for significant differences between treatments. The test design was based on Bray-Curtis similarities, the partial sum of squares type III, 999 permutations under a reduced model of raw data using Monte-Carlo simulations, and followed by pair-wise tests. Type III (partial) sums of squares were chosen as the most conservative model in which the order that terms are fitted is not important (Anderson et al., 2008). Permutations under a reduced model were selected as plastic treatment and time were the two factors. Moreover, Permutational analysis of multivariate dispersions (PERMDISP) (Anderson, 2006) was used to test for heterogeneity of community structure in a priori groups.

The effect of environmental parameters on the NSW community structure was assessed within all treatments using distance-based linear models (DISTLM). DISTLM procedure was used to identify the contribution of individual variables to explain variation in bacterial community datasets. The DISTLM routine was based on the Akaike Information Criterion (AIC) using stepwise selection procedure and 999 permutations. Step-wise selection was chosen as it allows for both the addition and removal of a term to the model at each step (Anderson et al., 2008). AIC selection was chosen as the method to create the most parsimonious model to determine the relative importance of all variables in explaining community variation that explained the most variations in community data. Furthermore, the 'Best' model-building approach was employed to examine the value ( $R^2$ ) of all possible combination of variables that resulted in the strongest correlation with the patterns of the bacterial community in all treatments. Distance-based redundancy analysis (dbRDA) plot was used to investigate the relationship between the best-correlated variables among all the plastic treatment communities. The vectors in the dbRDA plot are proportional to their contribution to the total variation. Each vector begins at the centre of the circle (the origin) and ends in the coordinates (x and y). The length and direction of each vector indicate the strength and sign of the relationship between the given variable and the dbRDA axes.

## 2.3 Results

### 2.3.1 Physico-Chemical Characterisation of Seawater

#### 2.3.1.1 Seawater pH

Figure 2.3.1a shows that the pH of the control, PET and PVC treated NSW demonstrated no difference in pH throughout this study and stayed at  $7.5 \pm 0.1$ . However, an increase in the pH to 8.05 and 8.28 for HDPE and LDPE treated experiments was observed at 512 hr after a decrease to 7.13 and 7.29 at 64 and 32 hrs, respectively. Similarly, the addition of PLA showed no change to the pH except for a small decrease to 7.23 at 64 hr, which then increased to 8.2 at 512 hr. In the PVOH treatment, the pH started to decrease at 16 hr point and gradually dropped to 6.3 till 128 hr. It was then increased to 7.0 when measured at 256 hr and peaked at 512 hr with a maximum of 7.45. The most notable change in the pH was observed in the PHB/PHV treatment. The pH dropped to 5.7 by 512 hr after initially started to decrease from 7.17 at 32 hr (Figure 2.3.1a). A repeated measures ANOVA of pH showed that it changed within the sampling intervals ( $F_{(9, 20)} = 31.49$ ,  $p < 0.05$ ). Posthoc Fisher's LSD test showed significant differences between the control and all the biodegradable plastic (PVOH, PLA and PHB/PHV) treatments ( $p < 0.05$ ). However, the pH only changed significantly between control and LDPE treatment ( $p = 0.036$ ) among the synthetic plastics.

#### 2.3.1.2 DOC, DIC and TN Determination

Figure 2.3.1b illustrates the seawater DOC values for each NSW treatment during the time course of the experiment. The DOC concentration of synthetic plastic (PET, LDPE, HDPE and PVC) treatments increased initially when plastics were added to the seawater. The highest DOC concentrations for PET (23.26 mg/L) and LDPE (14.63 mg/L) were measured at 4 hr; whilst the maximum DOC for HDPE (9.16 mg/L) and PVC (19.17 mg/L) treatments was observed at 2 and 8 hr respectively. Thereafter, the concentration gradually decreased until the end of the experiment shown in Figure 2.3.1b. Similarly, among the biodegradable plastics, PLA and PHB/PHV treated NSW showed an initial gradual increase in DOC concentrations. However, the DOC measurements for these plastic treatments continued to increase over time till the end of experiment and the DOC concentration was 18.22 and 118.98 mg/L, respectively at 512 hrs. The maximum increase in the DOC concentration was measured for PVOH treatment as it reached to 158.71 mg/L at 512 hr (Figure 2.3.1b). A repeated measures ANOVA of the DOC showed that the concentration changed within the

sampling intervals ( $F_{(9, 20)} = 942.36$ ,  $p < 0.05$ ). Posthoc Fisher's LSD test showed significant differences between the control and all the plastic ( $p < 0.05$ )

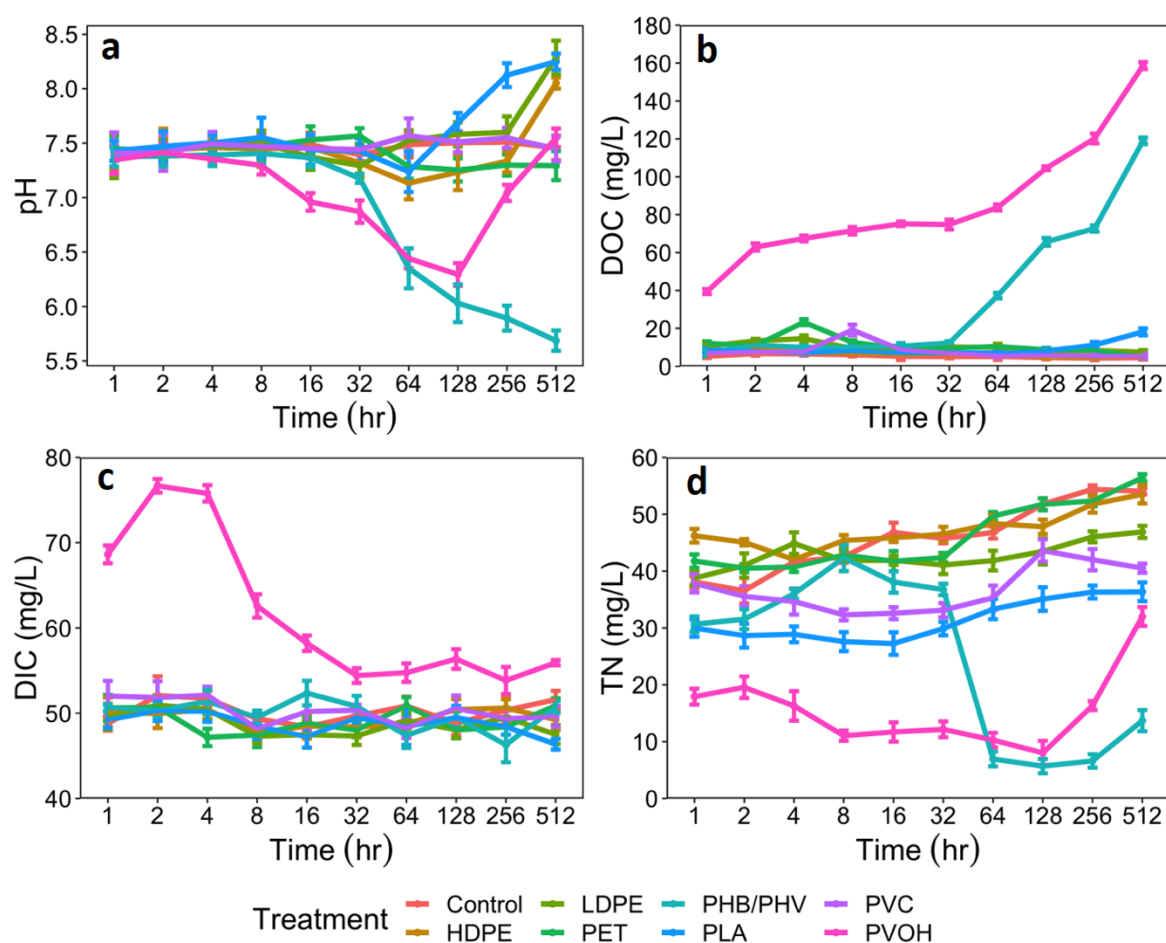


Figure 2.3.1 Profiles of physico-chemical parameters of NSW during plastic treatments: (a) pH, (b) DOC, (c) DIC and (d) TN. Each parameter plotted over sampling time for three replicates. Error bars represent the standard deviation for the average measurement at each sampling point.

The DIC and TN concentrations were also analysed at each sampling point (Figure 2.3.1 c,d). Initially, the DIC concentration in the PVOH treated seawater was higher than the control and peaked at 2 hr with a maximum of 76.66 mg/L. The concentration then gradually dropped by the end of the experiment to 55.93 mg/L at 512 hr (Figure 2.3.1c). There were evidently some variations among all other treatments and the control but the DIC concentration remained between 47.17 to 51.83 mg/L. A repeated measures ANOVA of the DIC showed that the concentration changed within the sampling intervals ( $F_{(9, 20)} = 45.54$ ,  $p < 0.05$ ). Posthoc Fisher's LSD test showed significant differences between the control and the PET, HDPE, LDPE, PVOH and PLA treatments ( $p < 0.05$ )

The TN concentration of the synthetic plastic (PET, LDPE and HDPE) treated experiments showed a similar trend except for PVC treatment (Figure 2.3.1d). The TN concentration in PET, HDPE and LDPE treatments fluctuated slightly during the first 4 hrs, but a gradual

increase in the concentration was observed between 64 and 512 hrs. Conversely, the TN of PVC treated NSW decreased between 4 and 64 hrs then rapidly increased to 43.61 mg/L at 128 hr. This eventually decreased to 40.52 mg/L at 512 hr.

In the biodegradable plastic treatments, relatively lower TN concentrations were observed initially (29.98, 30.66 and 17.92 mg/L for PLA, PHB/PHV and PVOH respectively at 1 hr). The TN concentration of PLA treatment remained steady to 32 hr, where it then progressively increased to 36.35 mg/L at 512 hr. However, the PHB/PHV treatment showed a maximum increase to 42.32 mg/L between 2 and 8 hrs. The TN then dropped to 6.96 mg/L at 64 hr and maintained between 5.7 to 6.62 mg/L till 256 hr and then slowly increased to 13.7 mg/L by the end of the experiment. In contrast, an opposite trend was observed in PVOH treatment where the TN concentration began to increase at 256 hr and reached to maximum 32.01 mg/L at 512 hr after gradually decreasing to 8.02 mg/L till 128 hr (Figure 2.3.1d). A repeated measures ANOVA of the TN showed that the concentration changed within the sampling intervals ( $F_{(9, 20)} = 61.66, p < 0.05$ ). Posthoc Fisher's LSD test showed significant differences between the control and the LDPE, PVC, PVOH, PLA and PHB/PHV treatments ( $p < 0.05$ ).

## **2.3.2 Microbial Community Analysis**

### **2.3.2.1 Seawater Cell Enumerations**

Total cell counts within the control and treated seawater microcosms were measured during the sampling period to further assess the influence of plastics on the microbial community. As shown in

Figure 2.3.2, the microbial cell density across all the treatments was between  $4.3$  and  $5.2 \times 10^6$  cells per mL after 1 hr of exposure to plastic. The cell counts in the untreated control seawater were comparatively low to all the treated samples throughout the duration of the experiment. A repeated measure analysis showed that the difference of the cell abundances between all plastic treatments and the control were significant ( $F_{(7,16)} = 50.92, p < 0.05$ ) except PET ( $p = 0.056$ ). The maximum cell abundances measured within PET and PVC treatment were  $8.9 \times 10^6$  and  $1 \times 10^7$  cells per mL respectively, with no clear trend. In the LDPE and HDPE treatments, the cell counts showed increases at 16 and 32 hr ( $6.9$  and  $8.3 \times 10^6$  cells per mL respectively), followed by a steady increase until 64 hr and then a gradual decrease in cell density. However, an increase in cell number to  $9.7 \times 10^6$  cells per mL was observed at 512 hr in the HDPE treatment. In general, cell abundances were higher in biodegradable

plastic compared to synthetic plastic treatment and the control. The PVOH and PHB/PHV treatments showed the most significant cells increase (Posthoc Fisher's LSD tests,  $p < 0.05$ ). The cell abundances reached maximum values of  $1.4 \times 10^7$  (at 256 hr) and  $2.1 \times 10^8$  (at 512 h) cells per mL for PVOH and PHB/PHV treatments after the initial increase at 8 and 16 hrs respectively. However, the lowest cell counts were measured ( $1 \times 10^7$  cells per mL) for PLA treatment as compared to other biodegradable plastics at the end of the experiment.

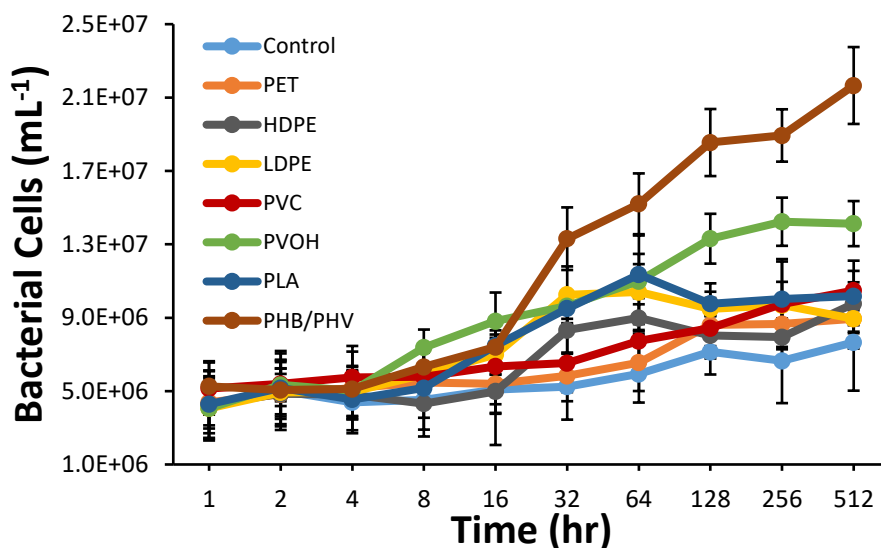


Figure 2.3.2 Bacterial cell numbers during plastic treatments. Bacterial cells ( $\text{mL}^{-1}$ ) plotted over sampling time for three replicates in seawater-plastic treatments. Error bars represent the standard deviation for the average cell count at each sampling point.

### 2.3.2.2 Microbial Diversity and Community Structuring based on T-RFLP Profiles

Overall, the  $\alpha$ -diversity indices (Shannon-Weiner  $H'$ ) for each treatment indicated that PET, HDPE, PVC and PLA treated NSW had the same diversity as the control. However, the diversity increased in LDPE and PVOH treatments whereas it decreased within PHB/PHV treated NSW (Figure 2.3.3 a; ANOVA,  $F_{(8, 81)} = 3.6319$ ,  $p < 0.001$ ). Moreover, for all of the treatments, the diversity declined initially (4-128 hrs) and then increased during the later period (from 256 to 512 hrs) (Figure 2.3.3 b; ANOVA,  $F_{(9, 70)} = 1.1994$ ,  $p > 0.001$ ).

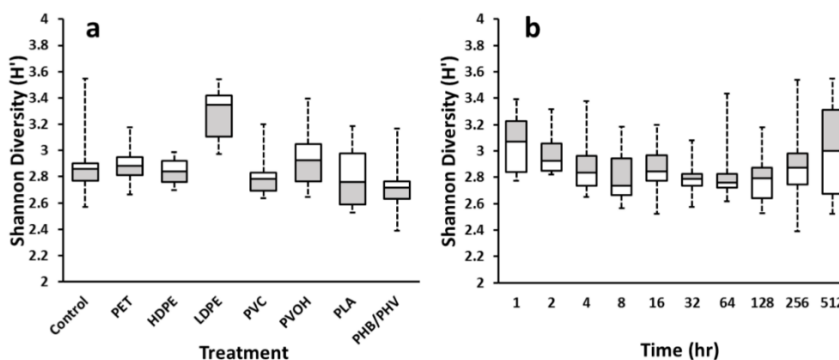


Figure 2.3.3 The Shannon-Weiner diversity indices are presented here as a boxplot comparing the  $\alpha$ -diversity between NSW and plastic treatments (a) and time points (b) measured in this study.

The bacterial communities identified in the controls ( $n = 3$ ) showed grouping among initial sampling points (1-64 hrs) in PCO ordination (Figure 2.3.4 a). However, variation between sampling points increased over time and at 512 hr, the community differed significantly (SIMPROF test,  $p < 0.05$ ). Similarly, for the PET treatment, the initial seawater bacterial community (1-4 hrs) clustered together but there was a shift in community structure at 8 hr. Subsequently, the communities between 16 and 512 hrs were maintained in a distinct cluster with an overall 60 % similarity (Figure 2.3.4 b). The PCO analysis of the HDPE treated bacterial community showed a clear separation between early (1-32 hrs) and late (64-512 hrs) sampling points (Figure 2.3.4 c). The differences in the bacterial composition were highly significant between these groupings (SIMPROF test,  $p < 0.05$ ). The LDPE treatment also showed a similar community structuring throughout the sampling periods (Figure 2.3.4 d). However, the variation was more significant (SIMPROF test,  $p < 0.05$ ) at 4 and 32 hr compared to HDPE treatment. For the PVC treated NSW, there was a greater dispersion of bacterial communities between 1-4 hrs indicating that the PVC addition affected their structuring process initially (Figure 2.3.4 e). However, the PCO then did not show pronounced separation among the rest of sampling communities (8-256 hrs) with the exception at 512 hr, which demonstrated a significant variation in the community (SIMPROF test,  $p < 0.05$ ).

The bacterial communities sampled between 1 and 128 hr showed clusters for the PVOH treatment (Figure 2.3.4 f), with an overlap in groupings indicating similarity between communities. However, the bacterial communities differed significantly (SIMPROF test,  $p < 0.05$ ) at both 256 and 512 hrs. The PCO ordination for the PLA treatment revealed that there were early shifts (2-4 hrs) in the communities (Figure 2.3.4 g) but the significant (SIMPROF test,  $p < 0.05$ ) variation was observed at 128 hrs. Moreover, the bacterial communities within PLA treatment further shifted away at 512 hr, indicating the gradual change in community composition at the end of the experiment. In the PHB/PHV treated experiments, the bacterial communities sampled during 16 to 256 hrs were distinct from those in the early (1-8 hrs) sampling period (Figure 2.3.4 h). However, further significant (SIMPROF test,  $p < 0.05$ ) variations in bacterial communities were also observed at 512 hr. SIMPER analysis showed that bacterial biofilm communities within the plastic treated seawater differed between 33.23 to 47.86 from each other, and between 30.45 to 41.84 % from the control seawater communities (Table. Appendix A.1). The overall similarity within all sampling point for each sample type (control and plastic treated seawater) remained between 75.39 to 88.36 % (Table. Appendix A.1).

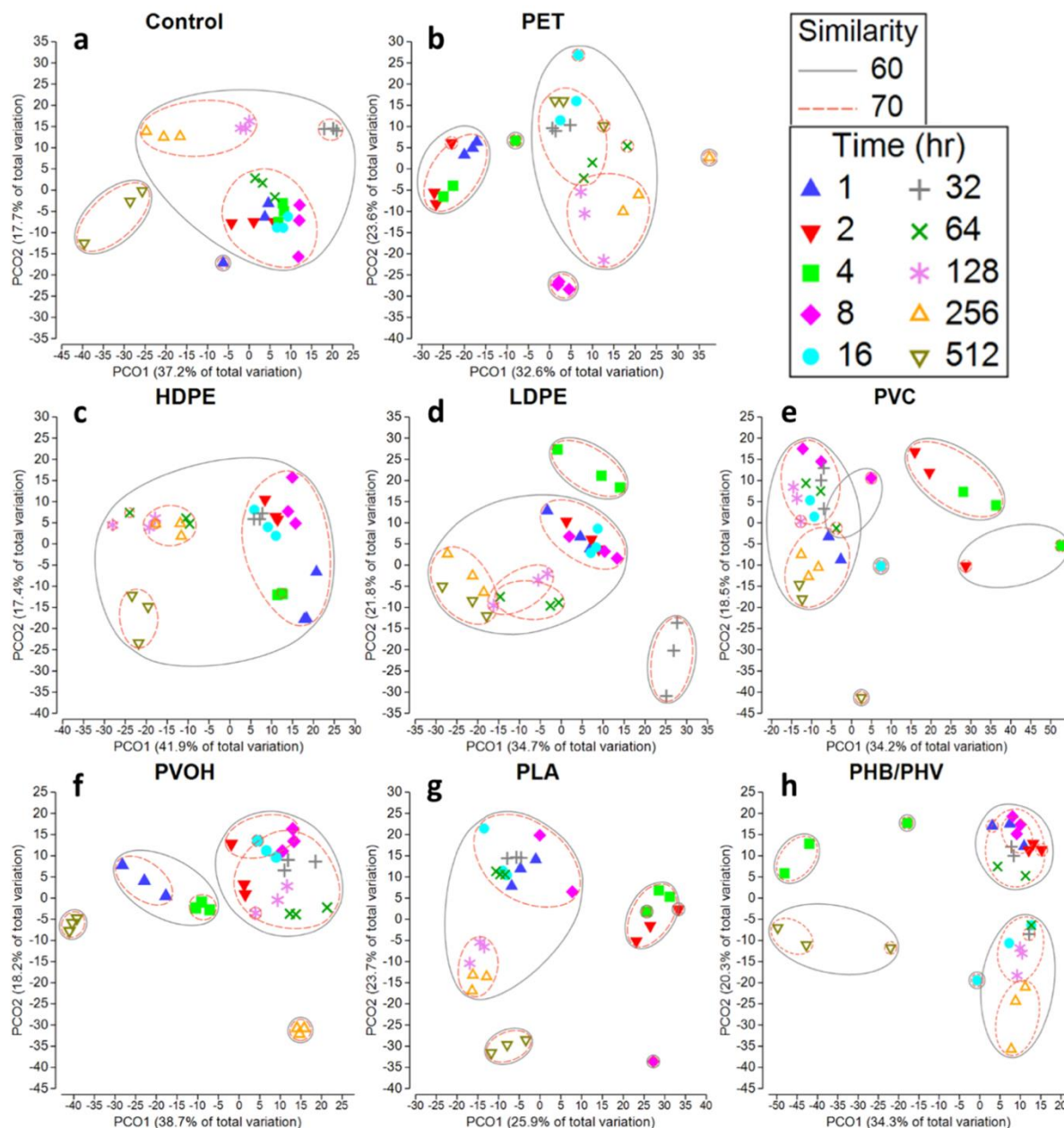


Figure 2.3.4 Principle Coordinate Ordinations (PCO) indicating variation in the bacterial community composition at ten sampling points. PCOs representing the similarity of the bacterial communities across all plastic treatments and the control, based on Bray-Curtis resemblance matrix. Circles indicate the percentage of similarity between samples based on cluster analysis and labels correspond to sampling time points in hours.

### 2.3.2.3 Substrate Specificity of the Seawater Microbiome

The control bacterial communities were compared with all the treatments to determine whether the plastic had a distinct effect on the seawater microbial community. PERMANOVA analysis of T-RFLP data revealed a significant difference in the treatments, a significant difference in the sampling time and a significant interaction between the control and the HDPE, LDPE, PVC, PVOH, PLA and PHB/PHV treated seawater communities (PERMANOVA,  $p < 0.05$ , Table 2.3.1). PERMANOVA test also revealed that the bacterial communities of the control and PET treated NSW were at the border of significance (PERMANOVA,  $p = 0.05$ ) when compared at the treatment level, indicating that the

difference in the average community compositions among these treatments were marginally equivalent. However, the communities significantly differed at sampling time and interaction between treatment and time (PERMANOVA,  $p < 0.05$  Table 2.3.1). Significant differences were also observed in possible-pair combinations, between control and the polymer-treated communities (Table. Appendix B.1). A separate test of dispersion using PERMDISP revealed that the differences among the plastic treated seawater bacterial communities to control were not driven by different within-system heterogeneities except for PLA and PHB/PHV treatments (Table. Appendix C.1).

Table 2.3.1 PERMANOVA test results of Bray-Curtis dissimilarities of bacterial community structure in relation to control vs plastic treated NSW. Tr = Treatment; Ti = Time; df = degree of freedom; SS = sum of squares; Pseudo-F = F value by permutation, boldface indicates statistical significance at  $P$  (perm)  $< 0.05$ ,  $p$ -values based on 999 permutations,  $P$  (MC) =  $p$ -values using Monte-Carlo tests,  $P$  (PERMDIP) =  $p$ -values from PERMDISP tests, calculated to centroids.

Treatments	Source of variation	df	SS	Pseudo-F	$P$ (perm)	perms	$P$ (MC)	$P$ (PERMDISP)
Control PET	Tr	1	1399	2.0155	<b>0.051</b>	999	0.058	0.092
	Ti	9	20879	6.8023	<b>0.001</b>	995	<b>0.001</b>	<b>0.011</b>
	TrxTi	9	16957	10.086	<b>0.001</b>	998	<b>0.001</b>	-
Control LDPE	Tr	1	1433.8	2.2785	<b>0.031</b>	998	<b>0.048</b>	0.531
	Ti	9	20879	6.8023	<b>0.001</b>	995	<b>0.001</b>	<b>0.009</b>
	TrxTi	9	10440	8.9615	<b>0.001</b>	997	<b>0.001</b>	-
Control HDPE	Tr	1	1792.7	3.0049	<b>0.012</b>	999	<b>0.011</b>	0.998
	Ti	9	17693	5.2561	<b>0.001</b>	998	<b>0.001</b>	0.181
	TrxTi	9	10036	6.49	<b>0.001</b>	998	<b>0.001</b>	-
Control PVC	Tr	1	2677.1	3.6676	<b>0.003</b>	999	<b>0.003</b>	0.085
	Ti	9	17876	3.6596	<b>0.001</b>	997	<b>0.001</b>	<b>0.009</b>
	TrxTi	9	16008	8.4183	<b>0.001</b>	995	<b>0.001</b>	-
Control PVOH	Tr	1	3786.8	5.1705	<b>0.001</b>	998	<b>0.001</b>	0.079
	Ti	9	22136	5.0965	<b>0.001</b>	995	<b>0.001</b>	<b>0.001</b>
	TrxTi	9	14108	10.057	<b>0.001</b>	999	<b>0.001</b>	-
Control PLA	Tr	1	1851.1	2.2229	<b>0.018</b>	998	<b>0.032</b>	<b>0.012</b>
	Ti	9	23073	4.7341	<b>0.001</b>	996	<b>0.001</b>	<b>0.005</b>
	TrxTi	9	15444	7.0169	<b>0.001</b>	997	<b>0.001</b>	-
Control PHB/PHV	Tr	1	5530	6.267	<b>0.001</b>	999	<b>0.002</b>	<b>0.003</b>
	Ti	9	21451	3.3799	<b>0.001</b>	996	<b>0.001</b>	<b>0.001</b>
	TrxTi	9	20041	9.1941	<b>0.001</b>	997	<b>0.001</b>	-

To facilitate the labelling of T-RFLP fragments, MiSeq analysis was carried out on the initially collected NSW sample using Illumina MiSeq technology. The dominated phyla in the initial NSW sample were *Proteobacteria* (90 %), *Bacteroidetes* (5.4 %) and *Actinobacteria* (1.5%). The NSW communities were further analysed at Family level and revealed that the seawater bacterial communities were dominated (relative abundance  $>1\%$  in each sample at each sampling point) by 15 Families (Figure 2.3.5). MiSeq sequences with a minimum of 80 % confidence at the family level (Wang et al., 2007) were chosen to



provide reliable identification of the bacterial community within the NSW sample. MiSeq analysis of the NSW sample showed that at the family level the bacterial community consisted of *Rhodobacteraceae*, *Gammaproteobacteria*, *Vibrionaceae* and *Alphaproteobacteria* making up 70 % of NSW bacterial community (Figure 2.3.5).

For identifying the taxonomic groups, in silico digestion of the MiSeq sequences was carried out to match the T-RFLP fragments. To account for any potential errors in the T-RFLP sequencing data, fragments representing the same taxon detected within approximately 4 base pairs of each other were grouped as the same bacterial species. Due to the variation in confidence levels provided by the MiSeq sequencing, the resolution of identification for each fragment varied, however resolution to family level was achieved for most fragments (Table 2.3.2).

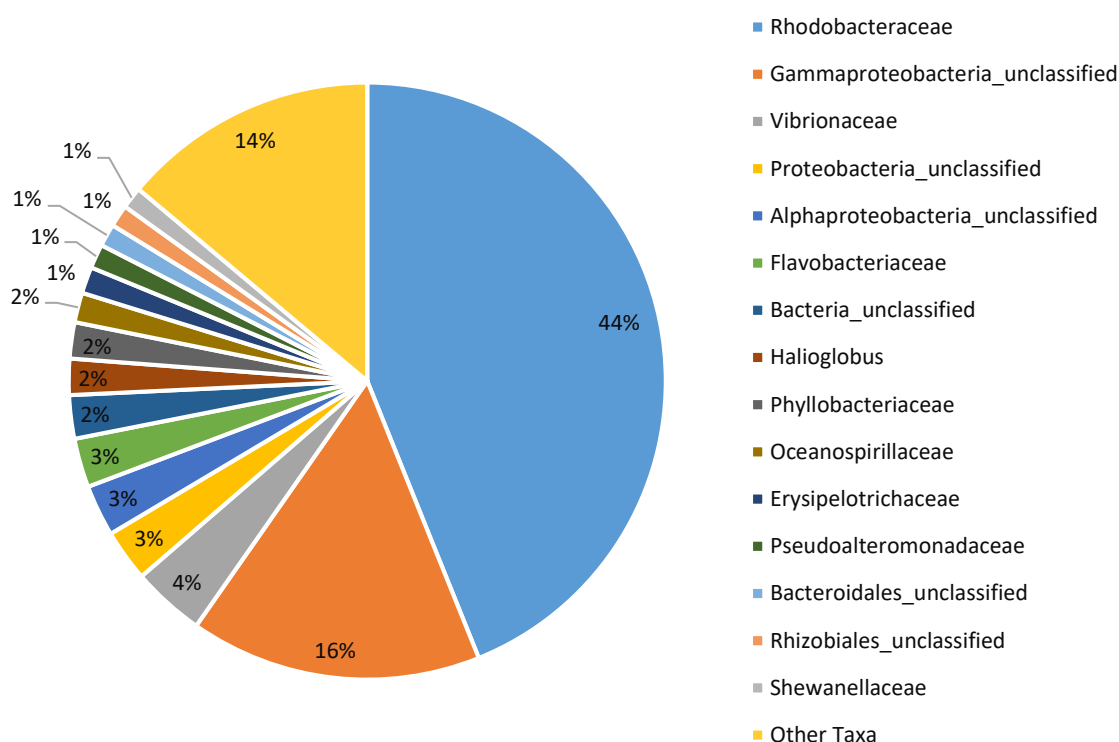


Figure 2.3.5 Bacterial community composition at the family-level classification of NSW sample collected for the study. Only families that represented at least 1% of the community are shown.

Table 2.3.2 Identification of T-RFLP fragments using MiSeq sequencing.

Fragment Size	Family	Fragment Size	Family	Fragment Size	Family
51-52	<i>Rhodobacteraceae</i>	108	<i>Desulfobulbaceae</i>	402-404	<i>Peptostreptococcaceae</i>
53-54	<i>Proteobacteria-unclaasified</i>	109	<i>Rhodobacteraceae</i>	409	<i>Cytophagales-unclaasified</i>
55-56	<i>Rhodobacteraceae</i>	111	<i>Intrasporangiaceae</i>	411	<i>Bacteria-unclassified</i>
58-60	<i>Gammaproteobacteria</i>	112	<i>Actinobacteria-unclaasified</i>	412	<i>Cytophagaceae</i>
61-63	<i>Rhodobacteraceae</i>	113-114	<i>Bacillaceae_1</i>	415	<i>Proteobacteria-unclaasified</i>
65-68	<i>Deltaproteobacteria-unclaasified</i>	117	<i>Hyphomonadaceae</i>	416-417	<i>Proteobacteria-unclaasified</i>
69	<i>Alteromonadales_incertae_sedis</i>	118-119	<i>Cytophagales-unclaasified</i>	418	<i>Campylobacteraceae</i>
72-73	<i>Rhizobiales</i>	120-121	<i>Bacteria-unclassified</i>	422-424	<i>Alteromonadaceae</i>
74	<i>Rhodobacteraceae</i>	122	<i>Alphaproteobacteria-unclaasified</i>	426	<i>Alteromonadaceae</i>
75	<i>Bacteria-unclassified</i>	123	<i>Rhodobacteraceae</i>	428	<i>Gammaproteobacteria-unclaasified</i>
76	<i>Reyranella</i>	125	<i>Gammaproteobacteria-unclaasified</i>	429	<i>Flavobacteriaceae</i>
77	<i>Desulfuromonadales-unclaasified</i>	126-127	<i>Flavobacteriaceae</i>	431	<i>Halioglobus</i>
78	<i>Actinobacteria-unclaasified</i>	129	<i>Proteobacteria-unclaasified</i>	432	<i>Pseudo</i>
79	<i>Actinobacteria-unclaasified</i>	130-132	<i>Bacteroidetes-unclaasified</i>	433	<i>alteromonadaceae</i>
81-83	<i>Iamiaceae</i>	133	<i>Bacteria-unclassified</i>	434	<i>Rhodothermaceae</i>
84	<i>Chromatiaceae</i>	135-136	<i>Gammaproteobacteria-unclaasified</i>	435	<i>Pseudoalteromonadaceae</i>
86	<i>Cytophagaceae</i>	138-139	<i>Peptostreptococcaceae</i>	436	<i>Gammaproteobacteria-unclaasified</i>
87	<i>Thiotrichaceae</i>	140	<i>Alteromonadaceae</i>	437	<i>Pseudomonadaceae</i>
88	<i>Oceanospirillaceae</i>	142-144	<i>Clostridiaceae_1</i>	439	<i>Colwelliaceae</i>
89	<i>Alteromonadaceae</i>	163-165	<i>Chloroflexi-unclaasified</i>	440	<i>Chromatiales</i>
90	<i>Flammeovirgaceae</i>	168-170	<i>Vibrionaceae</i>	442	<i>Shewanellaceae</i>
91	<i>Marinicella</i>	243-244	<i>Gammaproteobacteria-unclaasified</i>	443	<i>Moritellaceae</i>
93-94	<i>Erythrobacteraceae</i>	247	<i>Pseudoalteromonadaceae</i>	445-446	<i>Vibrionaceae</i>
					<i>Deltaproteobacteria-unclaasified</i>

97	<i>Erysipelotrichaceae</i>	253-255	<i>Oceanospirillaceae</i>	448-450	<i>Vibrionaceae</i>
99-100	<i>Microbacteriaceae</i>	365-367	<i>Alphaproteobacteria-</i> unclassified	452-456	<i>Gammaproteobacteria-</i> unclassified
101-103	<i>Nocardiaceae</i>	391	<i>Alphaproteobacteria-</i> unclassified	459-462	<i>Gammaproteobacteria-</i> unclassified
104	<i>Rhodobiaceae</i>	395	<i>Rhodobiaceae</i>	465-468	<i>Vibrionaceae</i>
105	<i>Alphaproteobacteria-</i> unclassified	399	<i>Rhizobiales-</i> unclassified	486-489	<i>Flavobacteriaceae</i>
106-107	<i>Bacteria-</i> unclassified	401	<i>Ectothiorhodospiraceae</i>		

The diversity of the bacterial communities in control NSW and their response to various plastic treatments (PET, HDPE, LDPE, PVC, PHOH, PLA and PHB/PHV) was assessed based on TRFs taxonomic identification (Figure 2.3.6). The bacterial community of the untreated control maintained a relatively consistent structure until 256 hr. The community was dominated by *Vibrionaceae* and *Gammaproteobacteria*, which accounted for > 60 % of the total abundance. Between 256 and 512 hrs, the microbial diversity decreased and the families, such as *Ectothiorhodospiraceae* and *Rhizobiales*, were not detected at 512 hr. At the end of the experiment, the major bacterial groups within the control NSW were *Flavobacteriaceae* (16 %), *Vibrionaceae* (15 %), *Gammaproteobacteria* (14 %), *Rhodobacteraceae* (9 %) along with unclassified *Proteobacteria* (8 %) and *Deltaproteobacteria* (5 %).

The bacterial communities in the PET treated microcosms showed an initial (1-8 hrs) increase within the families of *Flavobacteriaceae* and *Ectothiorhodospiraceae* and unclassified *Gammaproteobacteria* (collectively > 50 %). Moreover, members of the *Vibrionaceae* family increased between 8-16 hrs from approximately 2 to 60 % and subsequently remained dominant until the termination of the experiment. To a lesser extent, *Rhizobiales* and *Rhodobacteraceae* displayed a gradual change between 8 hr and 256 hr; an increase in the relative abundance of *Rhizobiales* (2 to 17 %) and a decrease in the relative abundance of *Rhodobacteraceae* (22 to 4 %).

In the HDPE and LDPE treatments, members of the families *Vibrionaceae* and *Gammaproteobacteria* dominated the community throughout the experiment. However, within the HDPE treatment, *Flavobacteriaceae* and *Rhizobiales* families gradually increased between 16 to 512 hr, from approximately 8 to 19% and 8% to 21 %, respectively. In contrast, the higher percentage of *Flammeovirgaceae* (15 %) and unclassified bacteria (24 %) was observed initially (4 hr) in LDPE treatment. Moreover, the members of the family *Ectothiorhodospiraceae* proliferated between 32 and 128 hr from approximately 3 to 21 % and then persisted for the duration of the experiment.

The microbial community structure of the PVC treated NSW was initially (1-4 hr) dominated by *Vibrionaceae*, *Gammaproteobacteria*, *Flavobacteriaceae* and *Flammeovirgaceae* – on average 28, 31, 17 and 16 % respectively. At 8 hr, the diversity decreased and several groups e.g. *Rhodobacteraceae*, *Flammeovirgaceae*, *Proteobacteria* and *Deltaproteobacteria* were not detected. Between the time points of 16-512 hrs, the members of family

*Flavobacteriaceae* bloomed from 10 to 41 % and a relative decrease was also observed within members of *Rhizobiales* and *Vibrionaceae* until the termination of incubations.

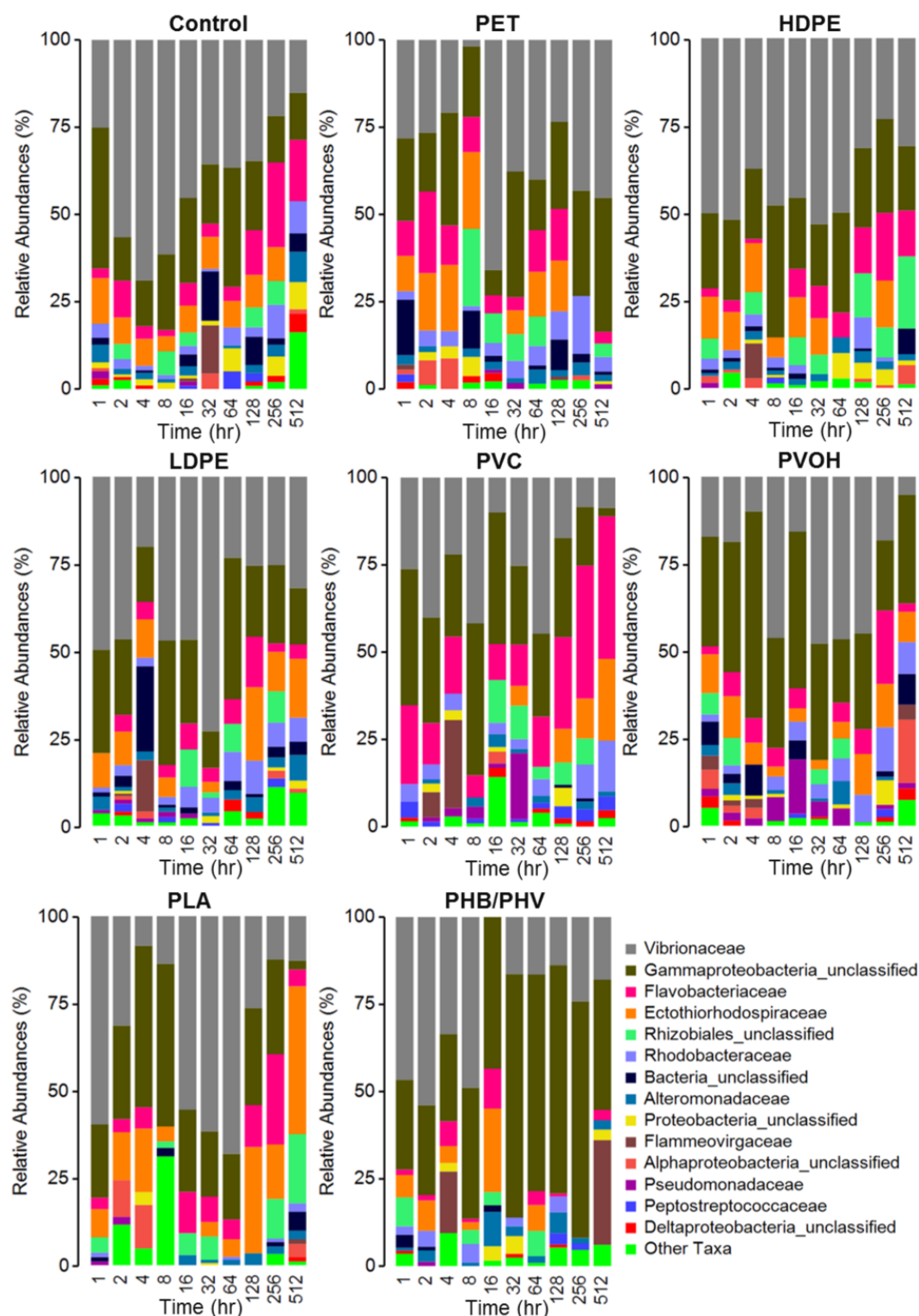


Figure 2.3.6 Bacterial community composition of NSW at family-level classification for each of the treatments over the 512 hrs experiment run. Only families representing at least 1% of the community are shown.

The marine bacterial community response in the biodegradable plastic treatments (PVOH, PLA and PHB/PHV) was different compared to the control and synthetic plastic treatments. Within 4 hrs of PVOH treatment, the community became strongly dominated by

*Gammaproteobacteria* (59 %), which subsequently prevailed as the key group for the duration of the experiment. Other notable bacterial groups included members of the family *Rhodobacteraceae* and *Ectothiorhodospiraceae* that proliferated to 13 and 12 % respectively at 128 hr. A short-lived increase in *Pseudomonadaceae* also occurred between 4 to 16 hr (2 to 16 %) and then progressively decreased thereafter. However, the most apparent shift in the bacterial diversity was observed at 512 hr, caused by an increase in *Alphaproteobacteria* (18 %), *Flammeovirgaceae* (4 %), *Deltaproteobacteria* (3 %) and unclassified bacteria (9 %).

Overall bacterial communities exhibited relatively low diversity in PLA treatment. Immediately after addition of PLA (1-16 hr), *Gammaproteobacteria*, *Ectothiorhodospiraceae* and *Vibrionaceae* were overwhelmingly the most abundant groups with approximate abundances of 45, 17 (at 8 hr) and 50 % (at 16 hr) respectively. The relative abundances of family *Vibrionaceae* further increased to 68 % at 64 hr then gradually decreased to 12 % until the end of incubation period. The members of *Flavobacteriaceae* and *Rhizobiales* also contributed to the diversity by constituting 10 % and 6 % and further increased to 25 % and 19 % respectively at the end of the experiment. Similarly, the *Ectothiorhodospiraceae* family increased to 31 % from the initial 8 % at 128 hr and the trend continued till 512 hr with the final value measured at 42 %. The members of *Rhizobiales* had also shown an increase in their abundance, constituting 11 % and 20 % respectively at 256 and 512 hr.

In PHB/PHV treatment, the bacterial community displayed a gradual change in taxonomic composition during the period of the experiment. The bacterial community of PHB/PHV treatment was initially (1-4 hrs) dominated by *Vibrionaceae* (40 %) and *Gammaproteobacteria* (31 %) as similarly observed in the PLA treatment. However, the members of *Gammaproteobacteria* progressively increased to 43 % at 16 hr and remained at relatively high abundance thereafter (66 %) till 256 hr. The members of family *Alteromonadaceae* were the other phyla that dominated within PHB/PHV treatment from 16 to 128 hr (approximately 9%). However, a more complex pattern of bacterial composition was observed at 512 hr as members within the family *Flammeovirgaceae* proliferated to 30 % with minor representation by *Alteromonadaceae* and *Proteobacteria* (3 and 4 % respectively) (Figure 2.3.6).

### 2.3.2.4 Relationship of the microbial communities with environmental variables

The environmental drivers of differences in marine bacterial community structure amongst plastic-treated seawater were identified using DISTLM procedure. The contribution of individual variables (DIC, DOC, TN, pH) was assessed by running a DISTLM routine, where the marginal tests in the DISTLM analysis (Table 2.3.3) report the proportion of the variation explained by each variable in each dataset, independent of other explanatory variables.

*Table 2.3.3 Results of DISTLM marginal tests. Results of marginal tests using a distance-based linear model (DISTLM) quantifying the relative contribution of chemical variables (DIC, DOC, TN and pH) on seawater bacterial community in all plastic treatments. Variables with significant values (P) were listed in bold. Prop.Var. the proportion of explained variance of corresponding seawater variable.*

Variables	Control		PET		LDPE		HDPE	
	P	Prop.Var.	P	Prop.Var.	P	Prop.Var.	P	Prop.Var.
DIC	0.742	0.0775	0.569	0.0914	0.604	0.0938	0.409	0.1059
DOC	<b>0.02</b>	0.2302	<b>0.005</b>	0.2993	0.499	0.1073	<b>0.004</b>	0.4221
TN	<b>0.044</b>	0.2013	<b>0.007</b>	0.2934	0.081	0.1830	<b>0.004</b>	0.41
pH	0.595	0.0940	0.164	0.1709	<b>0.003</b>	0.2932	0.265	0.1317
Variables	PVC		PVOH		PLA		PHB/PHV	
	P	Prop.Var.	P	Prop.Var.	P	Prop.Var.	P	Prop.Var.
DIC	0.73	0.0735	0.276	0.1389	0.223	0.1398	0.277	0.1440
DOC	<b>0.004</b>	0.3215	0.214	0.1470	0.125	0.1752	<b>0.043</b>	0.2501
TN	<b>0.025</b>	0.2648	<b>0.012</b>	0.2202	0.078	0.2010	0.54	0.0927
pH	0.647	0.0799	<b>0.019</b>	0.2088	<b>0.001</b>	0.3131	<b>0.039</b>	0.2584

Amongst the investigated physico-chemical variables, DOC and TN exhibited a significant effect on the bacterial community structures of the control, PET, HDPE and PVC treated seawater ( $p < 0.05$ ). Together these two variables explained 43 %, 59 %, 83 % and 58 % of total variations in the bacterial community datasets of each treatment, respectively. Whilst in the LDPE treatment, only pH was identified as a significant variable ( $p < 0.05$ ), which contributed alone to explain 29 % of the community variation. Similarly, in the biodegradable plastic treatments, the bacterial community was significantly influenced by pH ( $p < 0.05$ ), defining 20 %, 31 % and 25 % of the total variation within the PVOH, PLA and PHB/PHV treatments, respectively. However, the communities of PVOH and PHB/PHV treatments were also influenced by the individual effects of TN and DOC ( $p < 0.05$ ), contributing 22 % and 25 % of the variation respectively (Table 2.3.3).

The DISTLM ‘Best’ selection procedure was used for multivariate analyses to further narrow down the parameters that are most correlated with the community structures within all the treatments (Table 2.3.4). The overall fit for the model selecting all the variables for the control seawater was 0.53 (overall  $R^2$ ), with the Best procedure identifying DOC as the

variable that strongly correlated ( $R^2 = 0.23$ ) with the pattern of the bacterial community. Whilst a combination of all variables was identified as the Best solution for PET treated seawater bacterial community structuring ( $R^2 = 0.70$ ). For LDPE and HDPE treatment, the model selected pH as the strongly correlated (LDPE  $R^2 = 0.29$ ) variable but DOC and TN were also correlated with the community in the HDPE treatment (HDPE  $R^2 = 0.61$ ). Within the PVC treatment, DOC was the only variable that was significantly correlated with change ( $R^2 = 0.32$ ). The model selected DIC, DOC and TN as the strongly correlated three variables for PVOH treated community change ( $R^2 = 0.54$ ). Moreover, the pH was the most significant variable within PLA and PHB/PHV treatments that strongly correlated with the community however the Best model also included DIC and TN for PLA treatment (PLA  $R^2 = 0.57$ ; PHB/PHV  $R^2 = 0.26$ ).

*Table 2.3.4 Results of the DISTLM using 'Best' selection procedure showing the correlation coefficients between the Best all possible combinations of chemical parameters (marked by '✓') and the variability of seawater bacterial community in all plastic treatments.  $R^2$  values reported for corresponding significant variables and Overall Best model  $R^2$  values for all variables.*

Variables	Control	PET	LDPE	HDPE	PVC	PVOH	PLA	PHB/PHV
DIC		✓				✓	✓	
DOC	✓	✓		✓	✓	✓		✓
TN		✓		✓		✓	✓	
pH		✓	✓	✓			✓	✓
$R^2$	0.23	0.70	0.29	0.61	0.32	0.54	0.57	0.36
All variables selected	0.53	0.70	0.54	0.67	0.53	0.61	0.60	0.49
Overall $R^2$								

The top three variables indicated by Best analysis for all the treatments were visualised in the dbRDA plot (Figure 2.3.7); vectors were proportional to their contribution to the total variation. The most important variables, which explained the differences in the community composition for all treatments were DOC ( $P = 0.006$ ), TN ( $P = 0.001$ ) and pH ( $P = 0.014$ ). The dbRDA for chemical variables (Figure 2.3.7) showed a longitudinal separation of seawater bacterial communities of all treatments and the principal contributing chemical variables (DOC, TN and pH). The first two axes of the dbRDA plot of the all treatments explained 87.2 % of the variability in the fitted model and 9.4 % of the total variation in the bacterial community. This indicated that most of the salient patterns in the fitted model are captured. The vector overlays showed the relationship between the three variables found by the DistLM procedure. The first dbRDA axis was negative in relation to TN while the second axis showed a strong positive relationship with pH and DOC.



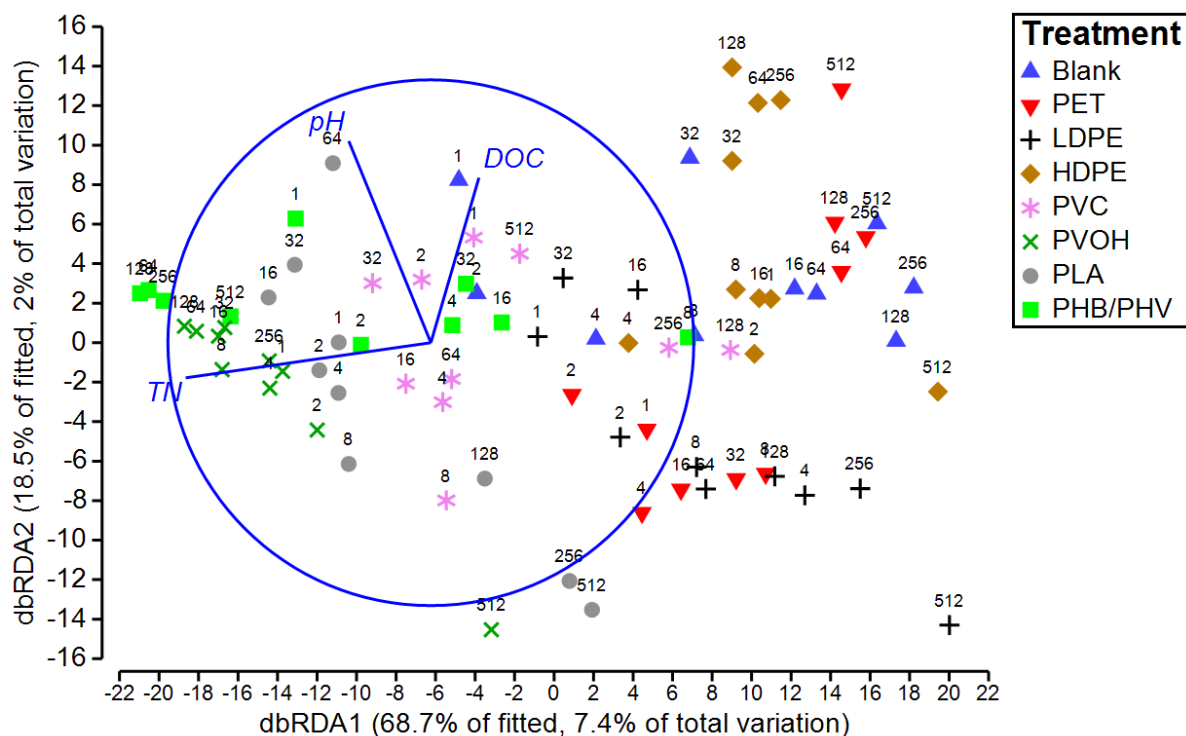


Figure 2.3.7 Distance-based redundancy analysis (dbRDA) plot to visualise DistLM results in 2-dimensional space for individual environmental variables. Vectors length is proportional to their contribution to the total variation and the circle denotes a correlation of 1.0.

## 2.4 Discussion

Microorganisms are well-known for their distinctive ability to quickly adapt to new ecological niches (Wiedenbeck and Cohan, 2011, Dutta and Paul, 2012). This adaptation applies also for the colonisation of marine plastics; an emerging artificial habitat in the ocean (Reisser et al., 2014). Plastic surfaces are immediately available for microbial colonisation (Harrison et al., 2014), which is well documented (Zettler et al., 2013, Oberbeckmann et al., 2014, De Tender et al., 2015). However, to date, less is known about the selective pressures plastics can have on the microbial biomass in the oceans. This study addressed the research gap and demonstrated that the plastics in seawater microcosm studies affected the diversity of marine bacterial communities, which could potentially affect the key nutrient cycling functions (e.g. carbon cycling) in the marine environment. Therefore, the results of this study provide important information for interpreting the effects of marine plastic debris on the microbial communities within areas of high plastic concentrations (e.g. Great Pacific garbage patch).

The addition of plastics affected the pH of the water in the treatments, which is an important physicochemical property of seawater. The pH has been implicated in governing the bacterial community composition in the marine environment, where small changes may favour distinct groups, possibly leading to compositional shifts in the community (Liu et al.,

2010). The most notable change was observed in the biodegradable plastic treatments. The pH within the PVOH and PHB/PHV treated seawater decreased significantly ( $p < 0.05$ ) to 6.3 and 5.7 respectively, from the initial value of 7.5. It is likely that the organic acids along with other metabolites produced as a result of microbial metabolism play an important role in the resultant pH decline (Sumbali and Mehrotra, 2009). The continuous increase in bacterial cell numbers (

Figure 2.3.2) within the PVOH and PHB/PHV treatments was also indicative of the production of these metabolites. The decrease in the pH may also be due to the solubility of the polymer, as a 5 % solution of PVOH exhibits a pH in the range of 5.0-6.5 (Ku and Lin, 2005). However, PHB/PHV is water-insoluble and resistant to hydrolytic degradation (Sudesh et al., 2000), thus it is more plausible that the PHB/PHV underwent microbial hydrolysis, which led to a progressive decrease in polymer chain length and ultimately the release of soluble oligomers (3-hydroxybutanoic acid and 3-hydroxypentanoic acid) (Kasuya et al., 2000) causing a decrease in the surrounding pH.

The present study demonstrated that irrespective of plastic type, the concentration of DOC increased initially when plastics were added to seawater and decreased thereafter for PET, LDPE, HDPE and PVC treatments till the end of the study. As all the plastics used in this study contained no additive or stabilisers, so it is reasonable to assume that the DOC leaching within the plastic treatments was not due to the presence of additives. However, micro and nano-particles can be derived either from plastic degradation or plastics surfaces which could then be released into the surrounding water (Suhrhoff and Scholz-Böttcher, 2016). Therefore, the DOC in this study included all the organic compounds released by the plastics in either dissolved form or fragmented particles detached from plastic materials. The DOC loss in the synthetic plastic treatments was in line with the other observations on the DOC leaching from plastics which estimated that 45 % of the leached DOC was progressively lost within 8 days after the first contact of the plastics with seawater (Romera-Castillo et al., 2018). It has been suggested that the loss could be due to either re-adsorption of DOC onto the plastics, the loss of unmeasured volatile organic compounds resulting from plastic degradation, or a combination of both (Romera-Castillo et al., 2018). Moreover, the decrease in the DOC concentrations could also be due to its utilisation by the marine bacteria as an energy source which in contrast to Romera-Castillo et al. (2018) study was immediately available for bacterial utilisation. Besides being utilised by marine bacteria, the DOC degrades through photolysis in the marine environment (Johannsson et al., 2017). This photo-degradation/oxidation process transforms DOC to change its bioavailability or even

oxidizes DOC directly into CO<sub>2</sub> (Xie et al., 2004, Patel-Sorrentino et al., 2004). However, the decline of DOC in our study cannot be attributed to photo-oxidation since the experiment was conducted in the dark.

In contrast to synthetic plastics, the concentration of DOC in the seawater treated with biodegradable plastics (PVOH, PLA and PHB/PHV) continued to increase during the study. This increase could be due to water-soluble products generated by biodegradation of these plastics. Sakai et al. (1998) and Matsumura et al. (1999) proposed a PVOH biodegradation pathway that yielded water-soluble acetic acid and methyl ketone products. Similarly, biodegradation of PLA and PHB/PHV by marine bacteria have been shown to produce monomers of lactic acid and 3-hydroxybutyric acid, respectively (Kasuya et al., 2000). However, the DOC concentration of PLA treated seawater was comparatively lower to that of PVOH and PHB/PHV treatment. This could be owing to the resistance of PLA to microbial attack when compared to other biodegradable plastics in marine conditions (Sashiwa et al., 2018). Thus, it has been shown that the DOC concentration started to increase late during the experiment (256 hr) in the PLA treatment. Another explanation for the increased DOC concentration in the biodegradable plastic treatments could be the increase in microbial biomass (

Figure 2.3.2). Marine microorganisms generally range from 0.02 to 200 µm in size (Cavicchioli et al., 2003). In this study, any microbial biomass less than 2.5 µm would be quantified as DOC since the seawater samples were only passed through 2.5 µm filter. The higher number of cells observed in PHB/PHV and PVOH treatments may be linked to a higher concentration of readily available biodegradation products. Thus, the higher concentration of these products might be acting as a nutrient source for the seawater bacterial community (Watanabe, 2001, López-Pérez et al., 2012).

The most apparent effect of plastics in the seawater microcosms was the rapid stimulation of bacterial growth. This rapid response is probably due to the immediate availability of DOC leaching from the plastics, which could be used by heterotrophic growth, without the requirement for any period of bacterial adaptation (Romera-Castillo et al., 2018). Moreover, the T-RFLP analysis demonstrated shifts in the structure of seawater bacterial communities over time. Within the synthetic plastics (PET, LDPE, HDPE and PVC) treatment, a transient compositional shift was detected initially during 1-32 hrs. This was followed by a second community shift at the later periods (64-512 hrs) of the treatments which resulted in strikingly different diversity patterns. The distinct early and late shifts within these

treatments could be attributable to the DOC bioavailability as the marine bacterial communities are strongly coupled with the concentration of DOC (Alonso-Sáez and Gasol, 2007, Traving et al., 2017a). Therefore, the shifts in DOC concentration induced changes in the bacterial community compositions. In the presence of biodegradable plastics (PVOH, PLA, and PHB/PHV), however, the first community shift occurred relatively early (1-8 hrs) as compared to synthetic plastic treatments. This different response pattern seems to be due to the initial higher DOC concentration within the biodegradable plastic treatments which continued to increase till the end of the experiment. Furthermore, a clear shift in the bacterial communities was observed during the mid-treatment period (16-128 hrs) which shifted further apart towards the end of study (256-512 hrs), suggesting that the higher release of DOC from biodegradable plastics selected for and against certain species and thus resulting in decreased bacterial diversity within all these treatments (Figure 2.3.3). This observation agrees well with the other reports on the response of the microbial community to the DOC, which found that addition of DOC shifted the coastal bacterial communities toward more copiotrophic taxa (Sipler et al., 2017, Traving et al., 2017a).

The plastics used in that study were in either pellet or powder (only PVOH) (Table 2.2.1) therefore were all negatively buoyant in the seawater microcosms. Although, several plastic types exhibit positive buoyancy when entering the oceans (Andrady, 2011, Woodall et al., 2014). In fact, it is estimated that about 60% of all produced plastic has a lower density than seawater (Andrady, 2011), since it is composed of the polymers polyethylene and polypropylene. However, after a period of weeks to months buoyant plastic can start to sink due to biofouling and particle attachment and thus exported to sediments and deep sea (Lobelle and Cunliffe, 2011, Pauli et al., 2017, Kooi et al., 2017). A recent study has reported the effect of plastics on sediment bacterial communities and concluded that the presence of microplastics alters sediment microbial community composition and nitrogen cycling processes (Seeley et al., 2020). In this chapter the hypothesis was that the presence of different plastics would affect the structure and composition of intact seawater communities (i.e. is seawater bacterial communities with plastics different from seawater communities without plastics?). Therefore, the main focus was to distinguish the community difference with respect to presence of plastics and not due to the varying properties of the plastic materials (e.g. density, hydrophobicity, surface area, etc).

To examine the communities and identify putative bacterial populations responding to plastics addition, individual populations (i.e., TRFs), which contributed significantly to the differences in community composition between the treatments were identified throughout

the experiment sampling periods. The sequencing data of seawater used for the study showed that members within the classes of *Gammaproteobacteria*, *Bacteroidetes* and *Alphaproteobacteria* dominated the seawater which is in line with the other observations on seawater bacterial assemblage (Zettler et al., 2013, De Tender et al., 2017a). However, in contrast to these studies, the abundance of *Gammaproteobacteria* was relatively high in the seawater samples which could be due to spatial and seasonal variation (Suh et al., 2015, Walsh et al., 2015). Moreover, the higher abundance of *Gammaproteobacteria* could also be associated with the reduced pH (7.5) of seawater, collected for this study. It is lower than that of the ocean pH which is constantly remained at or above 8.1 (Pearson and Palmer, 2000).

The bacterial communities stimulated by the synthetic polymer (PET, LDPE, HDPE and PVC) treatments only differed in terms of their relative abundances, rather than changes to the types of taxa. The communities were composed of a high proportion of Alpha- (*Rhodobacteraceae* and *Rhizobiales*) and *Gammaproteobacteria* (*Ectothiorhodospiraceae* and *Vibrionaceae*). The proliferation of these taxa could be explained by several genera belonging to the taxa that are known to be the primary colonisers of marine artificial surfaces (Lee et al., 2008). Comparatively, the late increase of *Bacteroidetes* (*Flavobacteriaceae*) within the PVC treatment suggested that this class could be categorised as secondary colonisers, which in agreement with the observations of Elifantz et al. (2013). The strong selection of these families within the synthetic plastic treatments suggests the use of these plastics as an energy source. Though the synthetic plastics are stable substrates and difficult to degrade but these bacterial groups are very diverse and possess the metabolic potential to degrade and assimilate complex carbon substrates (Carvalho et al., 2006, Le Borgne et al., 2008, Kostka et al., 2011, Gutierrez et al., 2014). The proliferation of *Alphaproteobacteria*, *Gammaproteobacteria* and *Bacteroidetes* within these treatments also suggest an active usage of DOC as a carbon source. The ability to metabolise DOC in seawater by the member of these taxa is well documented (Jennifer and James, 2004, Sipler et al., 2017).

A comparison of the bacterial communities within the seawater microcosms showed different dynamics when biodegradable plastics were added. The bacterial groups belonging to *Pseudomonadaceae*, *Rhodobacteraceae* and other unclassified *Alphaproteobacteria* proliferated most within PVOH treatment. Members of the groups *Pseudomonadaceae* and *Alphaproteobacteria* are known to be able to degrade PVOH (Kawai and Hu, 2009). Similarly, a marine bacterium *Thalassospira povalilytica* sp. belonging to the class *Alphaproteobacteria* was isolated in the PVOH biodegradation study (Nogi et al., 2014). In

contrast, the microbial community was dominated by *Rhizobiales* and *Ectothiorhodospiraceae* within the PLA treated seawater. The species belonging to these families have previously been isolated and used in PLA biodegradation studies (Sangwan and Wu, 2008, Sangwan et al., 2009), thus potentially providing an explanation for their dominance within the PLA treatment. Moreover, within the PHB/PHV treatment, the bacterial diversity reduced and it was mainly due to two reasons: (1) the temporal disappearance of certain bacterial groups (i.e. *Flavobacteriaceae* and *Rhodobacteriaceae*) and (2) the strong selection of PHB/PHV degrading marine bacteria (i.e. *Gammaproteobacteria*), which became particularly prevalent in the nutrient-rich PHB/PHV biodegradation studies (Mergaert et al., 1995, Volova et al., 2010).

Bacterial populations differ in their response to the substrates released from organic particles, and consequently, different particles may attract and select certain bacterial species (Traving et al., 2017a, Bižić-Ionescu et al., 2018). Similarly, the proliferation of *Alpha* and *Gammaproteobacteria* that was observed in the PVOH and PHB/PHV treatments could be due to the higher DOC release from these plastics (Figure 2.3.1b). This probably fostered the development of these fast-growing bacterial populations which were also found to be most responsive to the DOC additions (Sipler et al., 2017) and thus decreasing the pH within these treatments due to their metabolic activity (Sumbali and Mehrotra, 2009). Moreover, small changes in the pH have a direct effect on seawater bacterial community composition and the phylogenetic groups including *Gammaproteobacteria*, *Flavobacteriaceae* and *Rhodobacteraceae* respond most notably to shifts in pH (Krause et al., 2012). Therefore, it is also plausible that the variation in the pH contributed in the selection of *Alpha* and *Gammaproteobacteria* within biodegradable plastic treatments. However, Witt et al. (2011) argued that pH-dependent changes in bacterial diversity and composition are largely contributed by *Flavobacteriaceae*. In contrast, *Flavobacteriaceae* was not responsive to pH change in both PVOH and PHB/PHV treatments but within PLA treatment, there was a significant increase in *Flavobacteriaceae* after 64 hr which could be attributable to the pH change as the pH slightly decreased during 32-64 hr (Figure 2.3.1a). Moreover, members of the class *Gammaproteobacteria* (unclassified and *Vibrionaceae*) were the other prominent bacterial groups initially but their abundance diminished progressively over time, falling to its lowest proportion by 512 hr.

Various physico-chemical variables play important roles in shaping bacterial diversity and community structure (Agogué et al., 2011, Signori et al., 2014). Most of the tested marine physical and chemical properties were significantly correlated with bacterial community

composition within the plastic treatments. DOC, pH and TN were identified as top-ranked environmental variables from the DISTLM analysis (Figure 2.3.7), shaping the seawater bacterial community which was in agreement with the previous studies (Krause et al., 2012, Luria et al., 2017). However, DIC was not significant to bacterial community variations here but has been previously observed to have a strong influence on bacterial community structure in other studies (Witt et al., 2011, Meyer et al., 2016).

When considering the impacts that physico-chemical variables have on bacterial community structure, it is predicted that DOC leaching directly from plastics is likely to be the most important variable correlated to changes in community structure in seawater (Romera-Castillo et al., 2018). As expected, this study supports the observation as the bacterial community structure and diversity of the control, PET, HDPE, PVC, PVOH and PHB/PHV treatment significantly correlated with the DOC (Table 2.3.4). However, despite the observed change in DOC within LDPE and PLA treatment, no correlation was identified between community diversity and the DOC data. Whereas, change in pH was the main factor affecting the structure and diversity within these treatments when DOC concentration was not a limiting factor. This could be explained as temporal shifts in marine microbial community composition may arise due to a small variation in seawater pH (Meron et al., 2011, Meron et al., 2012, Krause et al., 2012). Furthermore, potential relationships between microbial diversity and nutrient availability, specifically TN, were also detected across bacterial communities of PET, HDPE and PVOH treatments. A negative relationship was observed between TN and DOC within these treatments (Figure 2.3.7) which implies that TN could be a limiting factor where DOC is available (Seekell et al., 2015).

## **2.5 Conclusions**

This chapter demonstrated the influence that plastics has on the seawater microbial community within microcosm experiments, and the parameters (DOC, pH and TN) defining the resultant community structuring. Using a controlled experiment with a range of plastics, this study showed that the bacterial assemblages differed significantly not only from the control but also between the plastics. The various plastics induced single-end shifts in the composition and diversity of the marine community. Moreover, the DOC increased within synthetic plastic treatments and was significantly correlated with the structuring of the bacterial community. However, the microbial activity within biodegradable plastic treatments was influenced by decrease in pH which will also play a role in defining the

community diversity. These microcosm experiments can only provide an abstraction of the complex ecology of a natural environmental system. However, this study represents the first quantitative and culture-independent assessment of the plastics to function as potential drivers of marine microbial community structuring and diversity which play an important role in various biochemical processes within oceans. The present results provide a starting point for research into the ecology and functions of microbial assemblages in areas with high plastic concentrations (gyers) and understanding how the plastics are influencing the foundation of the ocean's life support systems which may be being reshaped by continuous increase of plastic waste.



# **Chapter 3: Diversity and Dynamics of Microbial Community Succession on Synthetic Plastics in Seawater: A long-term Marine Exposure Study**

## **3.1 Introduction**

In Chapter 2 it was demonstrated that the presence of plastic materials in seawater induced major shifts in the composition and diversity of the microbial community. This was attributed to changes in the physio-chemical parameters of the water resulting from the breakdown of the material. Whilst Chapter 2 focused on the microbiology of the surrounding seawater, it is recognised that due to the longevity of plastic in the marine environment, this material also serves as a distinct and extensive surface for microbial colonisation (Zettler et al., 2013). However, there is a lack of ecological information about the formation and temporal dynamics of biofilm formation on synthetic plastics under comparable marine environments. Therefore, it is relevant to characterise the microbial communities living on plastics to identify microbes that interact and preferentially attach to plastics in marine environments. This Chapter looks to build on existing work by providing a better understanding of the impact of plastics on marine microbial life.

Microbial colonisation of plastic occurs relatively quickly (within one week) (Lobelle and Cunliffe, 2011, Harrison et al., 2014) in aquatic environments, a process which is no different to any other artificial surfaces (e.g. glass, steel and acryl) (Lee et al., 2008). Upon the release of a plastic item into the aquatic environment, a coating layer of organic and inorganic substances is formed (called “the conditioning film”) (Loeb and Neihof, 1975). This is then rapidly colonized by microorganisms, which form a biofilm on the surface (Cooksey and Wigglesworth-Cooksey, 1995). The resulting biofilms are functionally and phylogenetically diverse communities of bacteria, algae, protozoans, and fungi that are embedded within an exopolymeric substance matrix (EPS). They are collectively termed as a biofouling community, microbial assemblage, or periphyton (Donlan, 2002). (Davey and O'toole, 2000). These natural assemblages offer various advantages to microorganisms including: protection against toxic substances; metabolic cooperativity; possibilities for forming stable

consortia; horizontal gene exchange and nutrients accumulation (Flemming, 1998, Davey and O'toole, 2000).

In order to understand the ecological impacts of the plastic-attached biofilms, it is important to study how the microbial community develops over time and how the composition of the plastic affects the community dynamics and diversity. Some studies have already analysed the biofilms attached to marine plastics using culture-independent methods (Zettler et al., 2013, Oberbeckmann et al., 2014, Amaral-Zettler et al., 2015, De Tender et al., 2015, Bryant et al., 2016, Debroas et al., 2017). These studies have essentially focused on comparing the biofilm communities of plastic debris biofilms to the free-living bacteria in the sea. However, little is known about the temporal development and dynamics of marine biofilms, formed on chemically distinct synthetic plastics e.g. polyolefins, polyesters. The investigations of biofilm formation on plastic surfaces are scarce and the reconstruction of the mature biofilm based on randomly collected plastic debris was proven to be difficult due to their unknown origin, travel history and exposure duration (De Tender et al., 2017a). Only a few studies have so far described either the early (< 4 weeks) (Lobelle and Cunliffe, 2011, Harrison et al., 2014, Eich et al., 2015, Oberbeckmann et al., 2016) or the long term (> 6 months) biofilm formation (Webb et al., 2009, De Tender et al., 2017a) onto the selected plastics within marine environment.

This Chapter examines the influence of polymer type on the composition of marine biofilm communities when compared to indigenous free-living microbial inhabitants in seawater. High-density polyethylene, polyethylene terephthalate and polyvinyl chloride were selected for this study due to their cumulative share of 38 % in global polymer production (Geyer et al., 2017) and were also widely reported as major components of plastic debris in world's oceans (Barnes et al., 2009, Morét-Ferguson et al., 2010, Browne et al., 2011, Duhec et al., 2015).

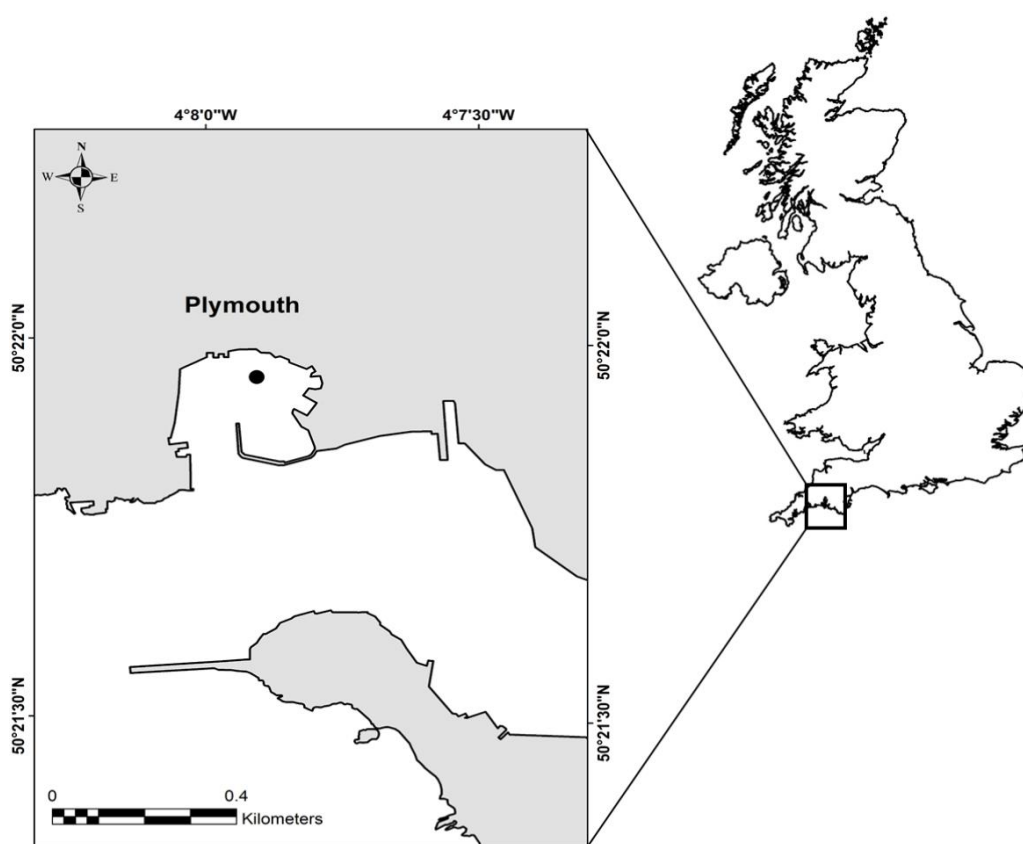
The specific aims of this Chapter are:

1. To investigate the long-term biofilm formation and progression on different synthetic polymers, in the natural marine environment;
2. To measure the temporal variations in the structure and diversity of plastic biofilms over time;
3. To identify the common genera within the plastic colonising bacterial assemblages.

## 3.2 Materials and Methods

### 3.2.1 Experimental Setup and Sampling

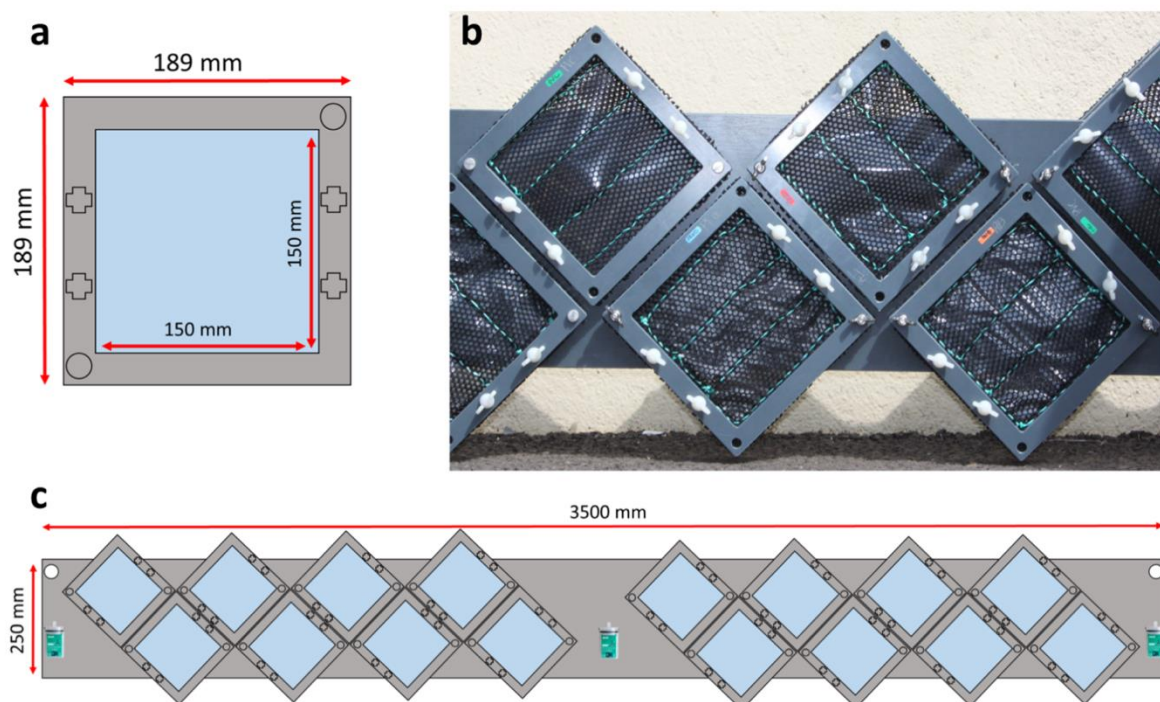
A long-term exposure experiment was designed to investigate the temporal dynamics of microbial biofilm communities attached to synthetic plastics in natural seawater. Three types of plastic films; high-density polyethylene (HDPE; thickness 0.01 mm), polyethylene terephthalate (PET; thickness 0.013 mm) and polyvinyl chloride (PVC; thickness 0.2 mm) (Goodfellow Cambridge Ltd, Huntingdon, UK) were submerged in the seawater at Queen Anne's Battery Marina (50°21'57.1"N 4°07'53.8"W) Plymouth, UK (Figure 3.2.1) over the period of 56 weeks from 17 July, 2017 to 13 August, 2018.



*Figure 3.2.1 Map of the exposure study site. The small black box denotes the regional location of the site within the UK. Location of field site at Plymouth, Devon, UK is shown in the magnified box. Grey colour represents land and white represents the sea. Circular marker denotes the sample deployment site.*

Three strips (140 mm by 40 mm) of each plastic type were placed in thick square PVC frames (189 × 189 mm) (Figure 3.2.2a). To avoid sample loss during the exposure period, each plastic film was further secured separately between nylon micromesh (mesh size = 4 mm) (Allplas Ltd, Letchworth, UK) (Figure 3.2.2b). 40 sample holder frames were then fixed on both sides of a supporting beam (3500 × 250 mm) (Figure 3.2.2c) and suspended approximately 2 m below the water's surface from a floating pontoon to stimulate exposure

and conditions of plastic at the near-surface of the oceans. Moreover, three HOBO Pendant<sup>®</sup> temperature/light loggers (UA-002-08) (Onset, MA USA) were also attached to the supporting beam to record the daily temperature and light intensity.



*Figure 3.2.2 The deployment structure used to expose plastics in natural marine water. (a) the plastic films holding frame, (b) three strips of each plastic type secured between the nylon micromesh within each holding frame, (c) the arrangement of the sample holding frames mounted on 15 mm thick PVC supporting beam.*

The plastic samples were periodically retrieved from the beam after 3, 6, 9, 12, 18, 30, 42 and 56 weeks. When taken out of the water the sample frames were kept in seawater at 4 °C within a thermoelectric cool box (Mobicool U32, Waeco, Bromborough, UK) for transit. Upon arrival in the laboratory, each plastic film was immediately removed from the frames and washed gently with sterile artificial seawater (ASW) to remove loosely attached material. The washed plastic films were then cut into smaller pieces (20 × 10 mm), half of which were stored at -20 °C for DNA extraction and the other half was then used for subsequent analyses. Seawater samples were also collected at 0 and 56 weeks as described in Chapter 2 for molecular analysis.

### 3.2.2 Biofilm Assays

A quantitative biofilm assay was used to measure biofilm formation on the plastic film surface using an existing protocol developed by Lobelle and Cunliffe (2011) with slight modifications. For this assay, samples (5 × 5 mm, n = 8, totalling 24 at each sampling point) were gently rinsed with sterile ASW and air-dried for 45 mins in a sterile 24-well cell culture plate separately. The plastic films were then stained with 1% (w/v) crystal violet (CV)

solution for at least 45 mins and washed three times with sterile ASW to remove the excess CV. The stained samples were air-dried for another 45 mins and then placed into a new cell culture plate where 1 mL of 95 % (v/v) ethanol was added in each well to solubilise the CV. The plate was incubated for 10 mins at room temperature and then the plastic films were removed. The solubilised CV solution was further diluted 10-fold in 95 % ethanol and then 200  $\mu$ L of diluted CV solution was added to a 96-well flat-bottom polystyrene micro-titre plate in triplicate. The optical density (OD) was then measured at 595 nm using a microplate absorbance reader (ELx 808 BioTek, Swindon, UK). To correct the background staining of crystal violet, the mean optical density at 595 nm of the negative control (95% ethanol) was subtracted from the mean optical density of the stained biofilm.

### **3.2.3 Scanning Electron Microscopy**

The qualitative assessment of biofilm formation on plastic films was investigated through scanning electron microscopy. The plastic samples ( $5 \times 5$  mm,  $n = 3$ ) were fixed in 2.5 % glutaraldehyde in 0.1 M Sorensen's buffer (pH 7.2) for 1 hour at room temperature and washed three times with 0.1 M phosphate buffer (pH 7.2). The films were then immersed in 1 % osmium tetroxide ( $\text{OsO}_4$ ) solution (0.25g  $\text{OsO}_4$  in 25 mL 0.1 M phosphate buffer) for another 1 hr and washed again three times with 0.1 M phosphate buffer. The samples were dehydrated in a graded (30, 50, 70, 90 and 100 %) ethanol for 10 min each and then within absolute ethanol ( $3\times$ ) for 15 min. The dehydrated samples were fixed on 12 mm aluminium stubs using carbon tape and then gold-coated (10 - 12 nm thickness) with an SC7640 Mini Sputter Coater (Quorum Technologies, Laughton, UK) in an argon atmosphere. The surface of the plastics was examined using a Field Emission Gun- Scanning electron Microscope (FEG-SEM) (ZEISS Supra; 55-VP; Zeiss Micro-imaging, Gottingen, Germany), which was operated with an accelerating voltage of 3 kV and a 7 - 10 mm working distance.

### **3.2.4 DNA Extraction and Next-Generation Sequencing**

DNA from the plastic biofilms was extracted using a modified protocol from Griffiths et al. (2000) (as described in Chapter 2). Briefly, plastic strips ( $5 \times 10$  mm,  $n = 3$ ) of each substrate type was transferred to 2 mL Lysing Matrix E tube (MP Biomedicals, OH, USA) containing a mixture of 0.5 mL of 5 % hexadecyltrimethylammonium bromide (CTAB) solution in 120 mM phosphate buffer (pH 8.0) and 0.5 mL of phenol:chloroform:isoamyl alcohol in the ratio of 25:24:1. The tube was shaken at  $5.5 \text{ m.s}^{-1}$  for 40 secs using an MP Fastprep-24 homogeniser (MP Biomedicals, OH, USA) and then centrifuged at  $16,000 \times g$  for 15 min at  $4^\circ\text{C}$ . The aqueous phase (supernatant) was then removed and an equal volume of chloroform

isoamyl alcohol (24:1) was added to remove residual phenol, followed by centrifugation at  $10,000 \times g$  for 10 min at 4 °C. The total genomic DNA was subsequently precipitated overnight at 4 °C using  $2 \times$  volume of 30 % polyethelene glycol solution, followed by centrifugation at  $14,000 \times g$  for 15 min at 4 °C. The pelleted DNA was then washed with ice-cold ethanol (70 %), air-dried and re-suspended in 50 µL of nuclease-free water (Invitrogen™, UK). Subsequent purification was conducted using GeneMATRIX universal DNA purification kit (EURx, Gdansk, Poland). Microbial DNA was also extracted from the indigenous seawater at the beginning and end of the study as described in Chapter 2: (Section 2.2.4.3) The molecular size and the purity of the DNA extracts were analysed by agarose gel electrophoresis (1%) and the DNA extraction yields were measured with a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE). The DNA extracts of all samples were stored at -20 °C until further use.

Barcoded amplicon sequencing of the 16S rRNA gene was performed to study the bacterial communities within the plastic biofilms. The V4 hypervariable region of the 16S rRNA gene was selected for amplification and subsequent sequencing. Prior to amplicon sequencing, the DNA samples were tested for the successful amplification of the V4 hypervariable region using 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') (Caporaso et al., 2011) primers. Amplification was carried out using 1 µL of template DNA in a 50 µL reaction mixture containing 250 nM of each primer, 5 µL of 5× Taq buffer, 2 mM MgCl<sub>2</sub>, 0.1 mM of each dNTP and 1.75 U of Taq (New England Biolabs, MA). PCR conditions were as follows: initial denaturation for 5 min at 94 °C followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing at 53 °C for 40 sec, extension at 72 °C for 1 min and a final extension at 72 °C for 5 min. All PCR products were verified on a 2 % (w/v) agarose gel (Sigma, Aldrich, Germany) by electrophoresis with a DNA marker, 2-Log DNA Ladder (New England Biolabs, MA). The PCR products were then purified using co-precipitant pink (Bioline, London, UK) protocol according to the manufacturer's instructions. Successfully tested samples were then sent to Molecular Research Laboratory (Texas, USA) for library preparation and sequencing by Illumina MiSeq platform using a 600 cycle (2×300 bp) v3 MiSeq reagent kit (20,000 average reads) following manufacture's guidelines.

### **3.2.5 MiSeq Data Processing**

Illumina MiSeq reads were joined and barcodes and sequences less than 150 base pair (bp) were clipped (pre-processing by Molecular Research LP, Texas, USA). Reads were further

processed in MOTHUR v1.40.5 (Schloss et al., 2009) according to MOTHUR MiSeq SOP (Kozich et al., 2013) (online access September 2018), which included removal of sequences with homopolymers or ambiguous bases (> 8 bases). The remaining sequences were then aligned against a non-redundant SILVA database v132 (Quast et al., 2013) and trimmed to a maximum length of 430 bp before chimera removal using VSEARCH (Rognes et al., 2016). The taxonomic identity of sequences was determined by comparing with the MOTHUR formatted ribosomal database project (RDP) database (v.16). Any sequence returned as unknown, chloroplast or mitochondrial were removed from further downstream analysis. Operational taxonomic units (OTUs) were defined after removal of singleton sequences, clustering at 3% divergence (97 % sequence identity) and thus a total of 1,303,605 classified sequences was obtained for bacteria and archaea. The archaea sequences were removed in the further analysis. Finally, subsampling to 25400 sequences per samples was performed to calculate the rarefaction curves and to eliminate the sampling biases during the subsequent diversity analysis.

### **3.2.6 Statistical Analysis**

All statistical analyses on bacterial community data were carried out using multivariate routines in the statistical software package PRIMER 7 (v7.0.13) with the add-on package PERMANOVA+ (Clarke and Gorley, 2015). All 26 samples were assigned to the corresponding substrate type (HDPE, PET, PVC and seawater) and sampling period (weeks 0, 3, 6, 9, 12, 18, 30, 42 and 56). Relative bacterial read abundance was calculated by dividing the number of bacterial reads by the number of bacterial reads for each sample. The OTUs with a minimum relative abundance of 0.1% in each substrate type were considered for further analysis. A core microbiome was calculated for the plastic-attached bacterial community. For this, the OTUs contributing relative abundances of >1% of the total community within all samples at each sampling point were designated as core microorganisms. Rarefaction curves were also generated to visualize OTU richness of 16S rRNA genes from samples. Rarefaction curves are a plot of the number of species as a function of the number of sampling events. Thus, when the curve plateaus and increased sampling events do not increase species richness, it is possible to infer that the environmental site has been sufficiently sampled to ensure full representation of the species present.

To visualize patterns in community composition, principal coordinate analysis (PCO) was performed using the Bray-Curtis similarity index (S17). All data were log-transformed to meet the assumptions of parametric analysis and to calculate the resemblance matrix. To test

for statistically significant variance among the biofilm communities attached to the different substrates and time, and their various interaction effects, PERMANOVA with fixed factors and 999 permutations at a significance level of  $p < 0.05$  was performed. The test design used the parameters already described in Chapter 2 (Section 2.2.5). Similarity percentage analysis (SIMPER) (Clarke, 1993) was used to calculate the total similarity within and dissimilarity between the different groups of substrates and seawater communities. Furthermore, quantitative biofilm data was compared against two factors: plastic types and exposure period, using mixed factorial repeated-measures ANOVA. Generalised linear model (GLM) with two-way interactions (plastic-type  $\times$  time (weeks)) was used to analyse within-subject (time) and between plastic-type difference during the experiment. These statistical analyses were conducted using IBM SPSS Statistics for Windows v. 22.0.

### **3.3 Results**

#### **3.3.1 Biofilm Formation and Quantitation**

Biofilm formation was visible on all the plastic types after the first sampling time point (3 weeks of exposure) and the formation increased throughout the experiment (Figure 3.3.1). Initially, the plastic films were sparsely covered and had loosely attached sediment particles. The coating became denser over time due to macrofoulants (e.g. mussels, tunicates) and algae, however, this development varied strongly with plastic-type.



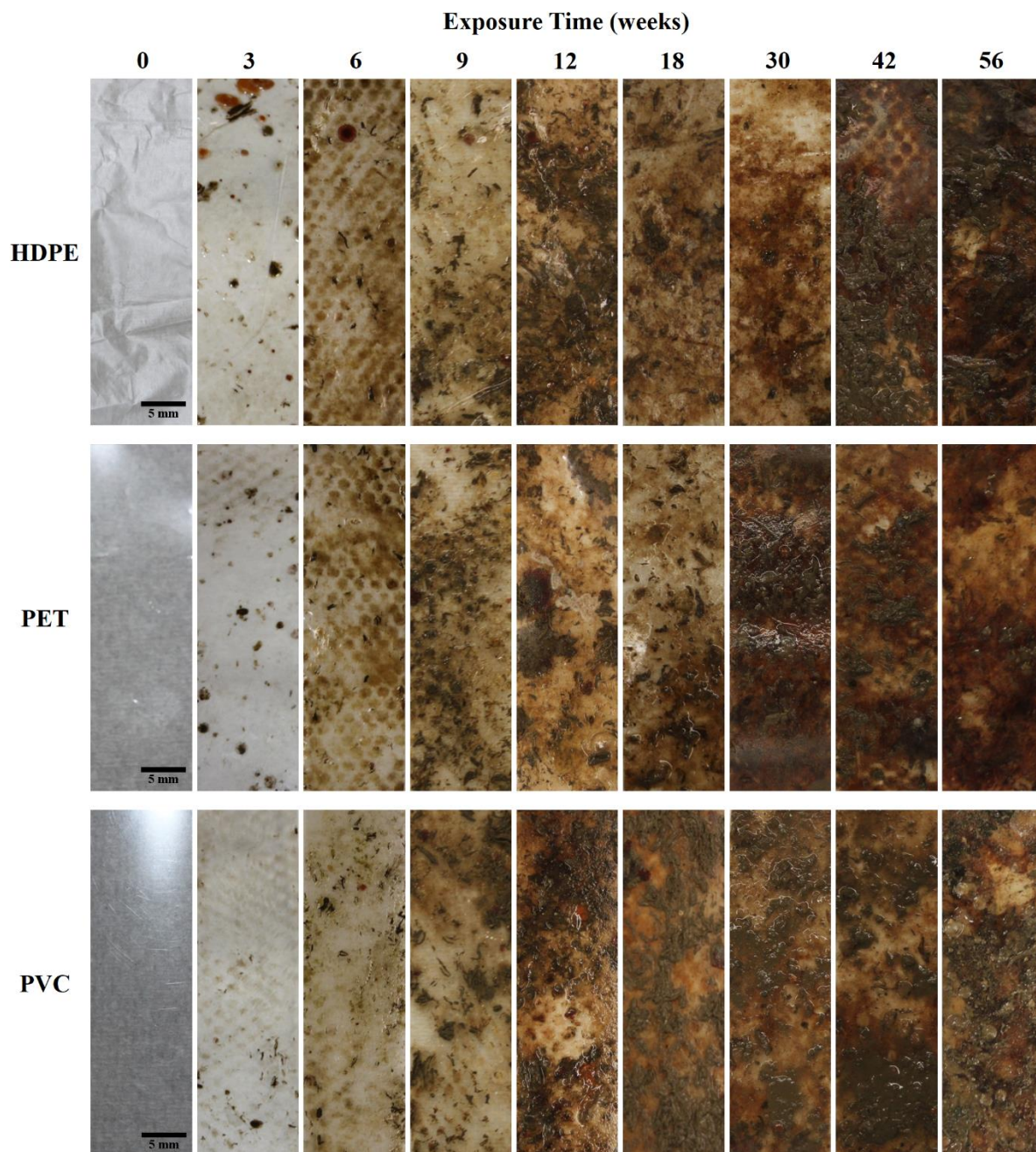


Figure 3.3.1 Biofilm formation on plastic films. The appearance of HDPE, PET and PVC films at each sampling point after submersion at the sea-end of Queen Anne's Battery Plymouth. (scale bar = 5 mm)

The biofilm was quantified using the CV staining assay after removing the loosely attached macrofoulants and sediment particles. A firmly attached biofilm was present on all plastic types after 3 weeks and the amount increased significantly over time (Figure 3.3.2; time:  $F_{(8, 168)} 174.43, p < 0.001$ , Table 3.3.1). For HDPE, the initial quantification of attached biofilm revealed that the OD increased to  $0.93 \pm 0.39$  after 3 weeks as compared to the control ( $OD = 0.35 \pm 0.03$ ). The biofilm formation continued to increase slightly over the sampling period and the OD was measured at was  $2.71 \pm 1.68$  after 30 weeks. However, a strong biofilm growth was observed during the late sampling intervals (42 and 56 weeks) of the exposure experiment and the OD value was  $3.92 \pm 1.37$  after 56 weeks (Figure 3.3.2 a). The increase

in the HDPE attached biofilm was found to be significant (HDPE,  $F_{(8, 63)} 47.39$ ,  $p < 0.001$  Table 3.3.1) over the sampling periods.

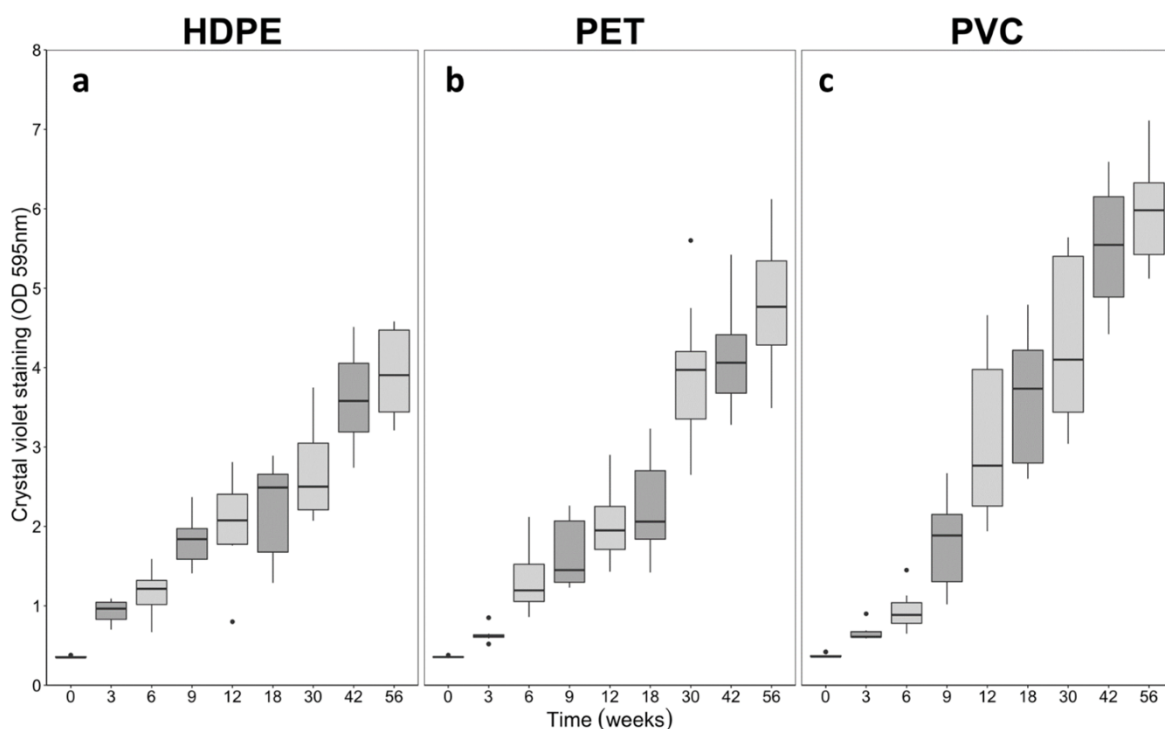


Figure 3.3.2 Boxplots representing the biofilm quantification by crystal violet staining assay on the plastic films at each sampling point. (a) HDPE (b) PET (c) PVC ( $n=8$ ). Time 0 represents the polymer without biofilm.

A significant (PET,  $F_{(8, 63)} 55.35$ ,  $p < 0.001$  Table 3.3.1) increase in the biofilm was also observed for PET films. The biofilm attached to the PET film was detected after 3 weeks of exposure ( $OD = 0.63 \pm 0.33$ ). A subsequent gradual increase in the biofilm formation was observed over time (3 to 18 weeks) and the OD value, measured for the amount of biofilm, was increased to  $2.22 \pm 1.81$  after 18 weeks. However, there was a strong increase in the amount of deposited biofilm after 30 weeks of submersion ( $OD = 3.95 \pm 0.91$ ) that continued to increase for the remainder of the experiment until 56 weeks ( $OD = 4.64 \pm 2.63$ , Figure 3.3.2 b).

Similarly, a temporal increase in the biofilm formation was measured for PVC films (Figure 3.3.2 c), however, the biofilm attachment was highest on PVC as compared to HDPE and PET. The average OD of PVC attached biofilm increased significantly (PVC,  $F_{(8, 63)} 71.94$ ,  $p < 0.001$  Table 3.3.1) from  $0.66 \pm 0.31$  (3 weeks) to  $5.96 \pm 1.99$  after 56 weeks. The quantification of the attached PVC biofilm corroborated the visual observations (Figure 3.3.1). Moreover, the repeated measures ANOVA results also showed that the amount of biofilm formed was significantly affected by the plastic-type (Table 3.3.1).

Table 3.3.1 Mixed factorial repeated measures ANOVA results of biofilm (OD 595nm) data in relation to plastic-type and exposure time. SS = Type III sum of squares; df = degree of freedom; F = F-value; p = significance value

Source	SS	df	Mean Square	F	p
HDPE	91.18	8	11.39	47.39	< 0.001
PET	161.79	8	20.22	55.35	< 0.001
PVC	283.23	8	35.40	71.94	< 0.001
Plastic type	26.20	2	13.10	31.03	< 0.001
Time (weeks)	500.88	8	62.61	174.43	< 0.001
Plastic type × Time (weeks)	35.31	16	2.21	6.15	< 0.001

### 3.3.2 Scanning Electron Microscopy of Microbial Biofilm on Plastics

Scanning electron microscopy was used to further examine the microbial biofilms on the three plastic types (HDPE, PET and PVC) at each sampling point. SEM analysis showed a morphologically diverse biofilm community, comprising of both prokaryotic and eukaryotic microorganisms, on all plastic types (Figure 3.3.3, Figure 3.3.4 and Figure 3.3.5). The examination of the HDPE film revealed the attachment of few rod-shaped bacteria after 3 weeks (Figure 3.3.3b). After 6 and 9 weeks, co-aggregation of bacterial cells were observed along with the attachment of intact pennate diatoms (Figure 3.3.3 c, d). Between 12 and 30 weeks, the biofilm was dominated with single-celled circular bacteria and the exopolymeric substance (EPS) was evident (Figure 3.3.3 e-g). However, the HDPE film was covered with a layer of organic matter and the biofilm consisted mostly of diatoms as well as broken diatom frustules towards the end of incubation (42 and 56-week) (Figure 3.3.3 h, i).

SEM images showed that the biofilm communities on the PET and HDPE samples were morphologically distinct (Figure 3.3.4). Following both 3 and 6 weeks of exposure in the seawater, several areas of the PET film were colonised by spherical-, rod- and filamentous-shaped bacterial cells (Figure 3.3.4 b, c). The surface of the PET film was dominated by clusters of bacterial cells and these cells appeared to be covered with exopolysaccharides, which were produced by the biofilm community between 9 and 18-weeks (Figure 3.3.4 d-f). After 30 weeks, the presence of centric diatoms and their remnants became more frequent (Figure 3.3.4 g). However, a well-developed biofilm containing a high amount of coccoid bacterial cells embedded in EPS along with diatoms and flagellates was observed on PET film at the end of the exposure experiment (Figure 3.3.4 h, i).

Inspection of the PVC films collected following 3-weeks of submersion in seawater by SEM revealed that the surface was heavily colonised with filamentous and rod-shaped bacteria (Figure 3.3.5b). The cell aggregations were more apparent at 6-weeks and presented cellular organisation in the form of microcolonies (Figure 3.3.5c). After 9 and 12-weeks of exposure, the PVC surface was associated with multispecies of microbial biofilms consisting of

pennate diatoms, as well as coccus-, rod-, and spiral-shaped cells (Figure 3.3.5 d, e). The biofilm cover increased at 18-weeks and much dense and tighter conglomerates were formed by pleomorphic bacterial cells (Figure 3.3.5 f). Similarly, cells of various phenotypes, especially with long filaments, were observed on 30 and 42-weeks samples (Figure 3.3.5 g, h). However, the PVC film was densely covered with sediment particles and organic matter (e.g. EPS) that prohibited the detection of microbial cells after 56 weeks of exposure (Figure 3.3.5i).

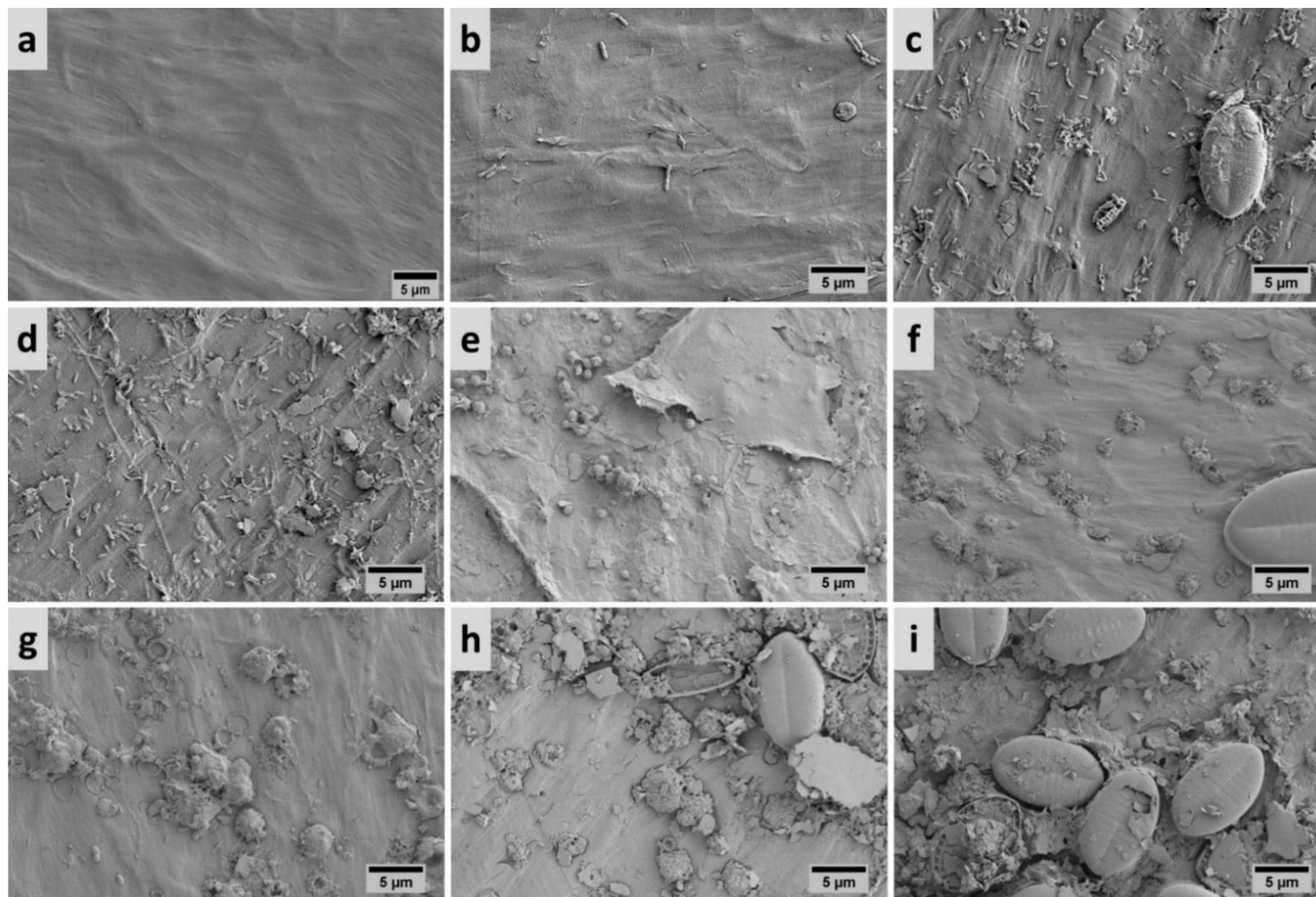


Figure 3.3.3 Scanning electron micrographs of microbial biofilm on plastic. Biofilm on HDPE films sampled after (a) control, (b) 3, (c) 6, (d) 9, (e) 12, (f) 18, (g) 30, (h) 42, (i) 56 -weeks exposure in marine water. Scale bar = 5  $\mu$ m.



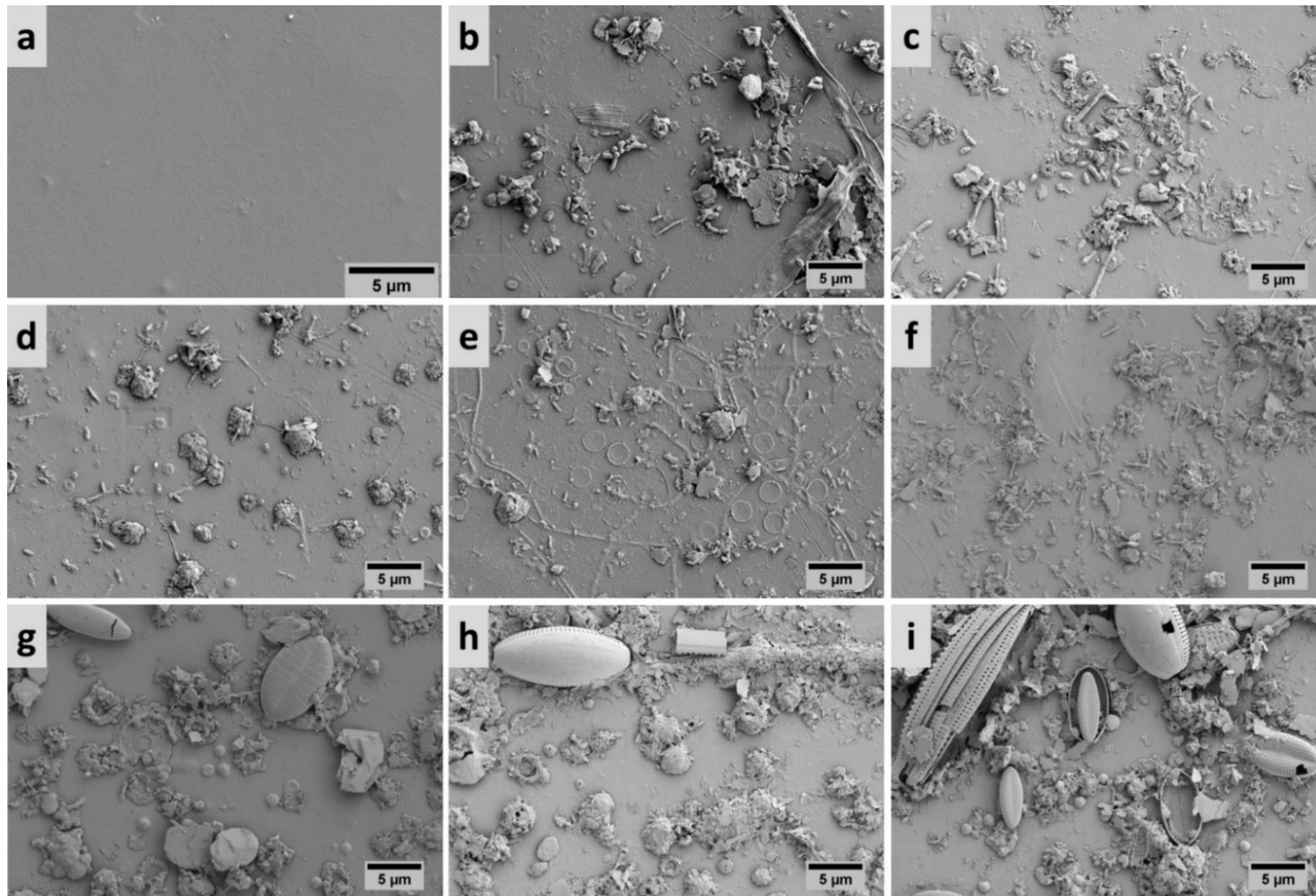


Figure 3.3.4 Scanning electron micrographs of microbial biofilm on plastic. Biofilm on PET films sampled after (a) control, (b) 3, (c) 6, (d) 9, (e) 12, (f) 18, (g) 30, (h) 42, (i) 56 -weeks exposure in marine water. Scale bar = 5 µm.

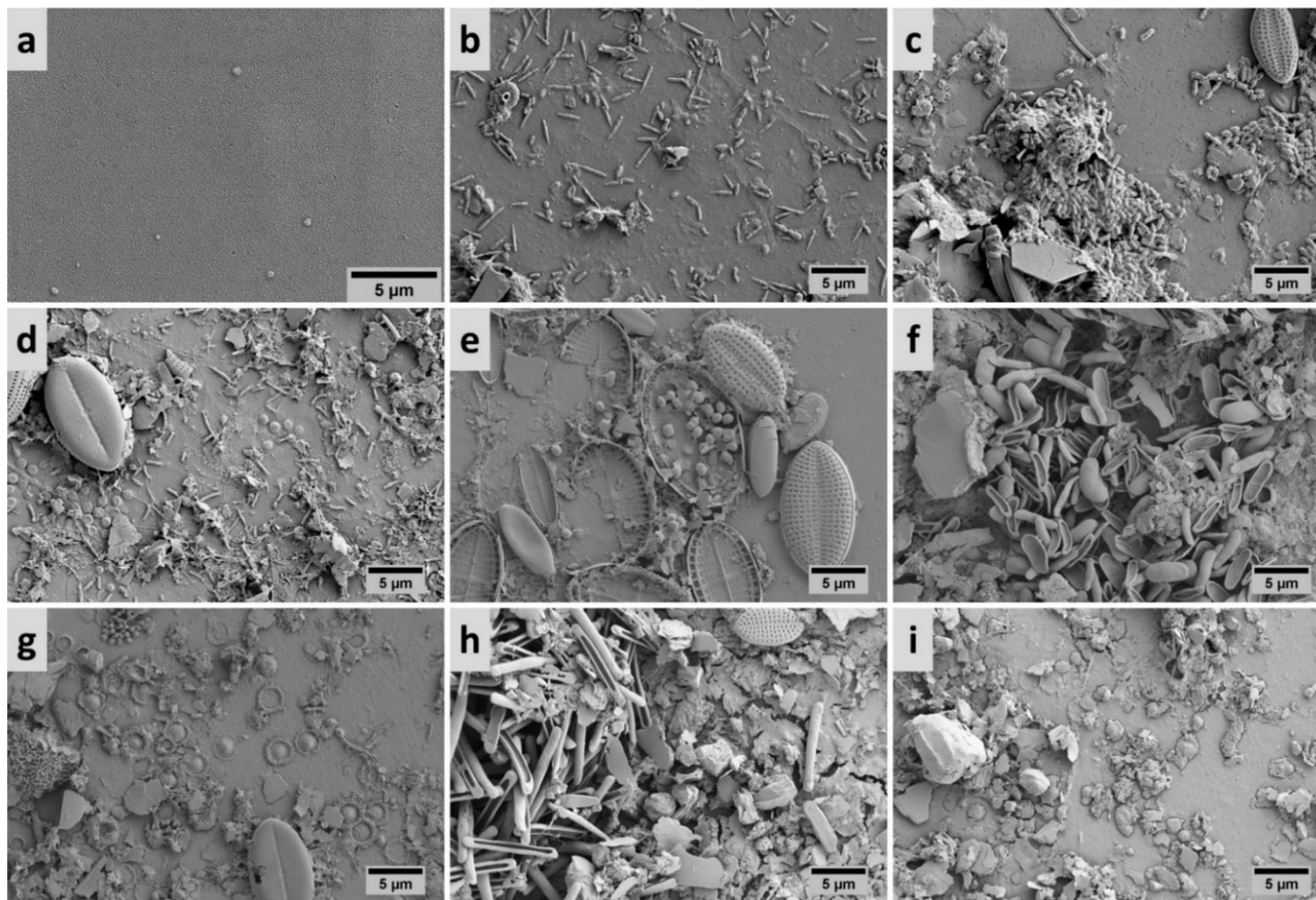


Figure 3.3.5 Scanning electron micrographs of microbial biofilm on plastic. Biofilm on PVC films sampled after (a) control, (b) 3, (c) 6, (d) 9, (e) 12, (f) 18, (g) 30, (h) 42, (i) 56 -week exposure in marine water. Scale bar = 5 µm.

### 3.3.3 Dynamics of Bacterial Community Composition

Barcoded 16S rRNA Illumina MiSeq technology was used to analyse the bacterial community associated with plastic films and the surrounding seawater at the test site. A total of 1,307,649 reads passed the quality control and ranged from 25,100 to 78,854 reads per sample. Figure 3.3.6 shows the bacterial community structure – at class-level classification – of biofilm composition formed on HDPE, PET and PVC at weeks 3, 6, 9, 12, 18, 30, 42 and 56, presented alongside the community of the surrounding seawater at 0 and 56 weeks. The bacterial community of the seawater maintained a relatively consistent structure at the beginning and end of the experiment (at 0 and 56 weeks respectively). The community was dominated by *Alpha*- and *Gammaproteobacteria* (58-68 %), with high contributions also by *Flavovacteriia* (5-12 %), *Actinobacteria* (5-10 %), *Planctomycetia* (2-4 %).

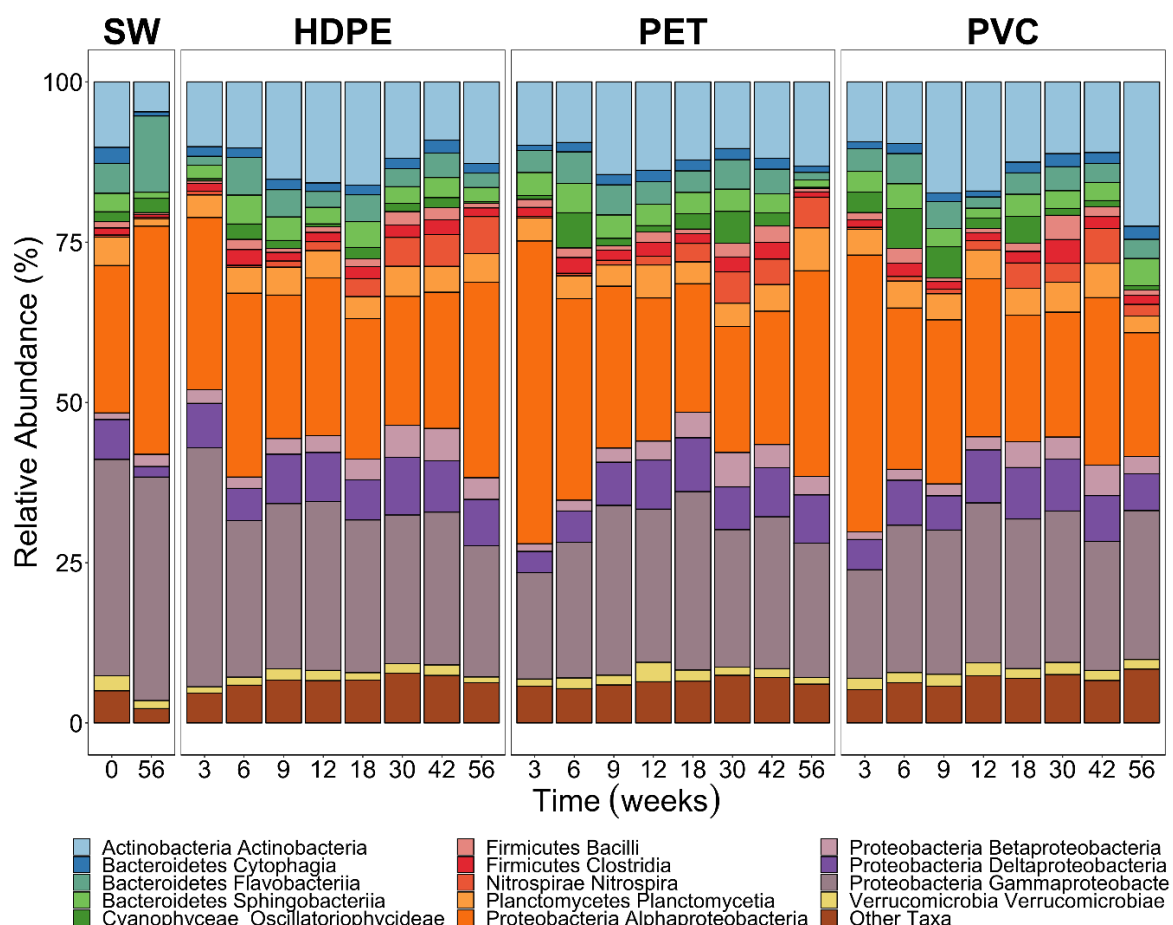


Figure 3.3.6 Bacterial community composition of plastics exposed to the marine environment and within surrounding seawater over time. Displayed are bacterial taxonomic classes with abundances of >0.1% in at least each sample at each sampling point.

For all the bacterial biofilm communities, the dominated phyla were *Proteobacteria* (53-74 %), *Actinobacteria* (5-16 %), *Bacteroidetes* (4-14 %) and *Planctomycetes* (2-8 %). The biofilm communities were further analysed at Class level and revealed that all of the biofilm communities were dominated (relative abundance >1% in each sample at each sampling



point) by 15 Classes (Figure 3.3.6). The most abundant classes were *Alpha*- (20-47 %), *Beta*- (5-2 %) *Delta*- (3-9 %) and *Gammaproteobacteria* (16-37 %). In addition, the taxonomic classes of *Actinobacteria* (9-22 %), *Planctomycetia* (3-6 %), *Flavobacteriia* (2-6 %), *Osillatoriphyceae* (1-6 %), *Nitrospira* (1-6 %) and *Sphingobacteria* (1-4 %) were also dominated in all biofilm communities.

All of the biofilm communities displayed a gradual change in taxonomic composition overtime (Figure 3.3.6). The change in biofilm communities on the HDPE films was caused by shifts in the abundance of particular bacterial groups. This included an increase in the relative abundance of *Alpha*- and *Betaproteobacteria* and *Bacteroidetes* (*flavobacteria*, *Sphingobacteria* and *cytophagia*) and a decrease in the relative abundance of *Gammaproteobacteria*. In contrast, the relative abundance of *Alphaproteobacteria* decreased on PET over the exposure period however, the relative abundance of *Gammaproteobacteria* increased initially (till 18 weeks) and then gradually decreased towards the end of the exposure period. Additionally, there was an increase in the relative abundance of *Delta*- and *Betaproteobacteria* and *Bacteroidetes*. Similarly, the biofilm on the PVC exhibited a decrease in the relative abundance of *Alphaproteobacteria* and increase in the relative abundance of *Actinobacteria* and *Nitrospira* (Figure 3.3.6). However, the relative abundance of *Gammaproteobacteria* remained steady throughout the experiment within the range of 17 to 22 %.

### 3.3.4 Structuring and Specificity of Bacterial Communities

To determine whether the microbial communities, which colonised the different plastic substrates, were distinct from that of within the seawater, the community structure of biofilms attached to the polymers was compared to seawater using  $\alpha$  diversity (rarefaction curves) and  $\beta$  diversity (Principal Coordinate Ordination PCO). The overall qualitative operational taxonomic units (OTUs; 97% sequence similarity) richness from the seawater and plastic biofilm appears to be high as rarefaction curves did not reach asymptote, even after 25000 sequences were examined for each sample (Figure. Appendix D.1, Figure. Appendix E.1, Figure. Appendix F.1 and Figure. Appendix G.1). Comparisons of 16S rRNA gene sequences rarefaction curves showed that the  $\alpha$ -diversity of the plastic biofilms was lower in the beginning of the exposure (Figure. Appendix E.1, Figure. Appendix F.1 and Figure. Appendix G.1). The diversity increased with the exposure period except for PVC biofilm for which  $\alpha$ -diversity decreased toward the end of exposure experiment (Figure. Appendix G.1). The communities attached to synthetic polymers and the seawater

communities clustered separately from each other (Figure 3.3.7). The 16S rRNA gene sequence comparisons showed significant differences between the seawater communities and those associated with synthetic polymers ( $p < 0.001$ ; PERMANOVA). The two factors: sample type (seawater, HDPE, PET and PVC) and the exposure time, both significantly ( $p < 0.001$ , PERMANOVA) affected the bacterial community composition, with highly significant interaction effects (all interaction effects  $p < 0.001$ ) between these two factors. Moreover, SIMPER analysis showed that the seawater bacterial communities had the average dissimilarity of  $>70\%$  with the plastic biofilm communities (Table 3.3.2), therefore, clustered separately from all plastic samples (Figure 3.3.7).

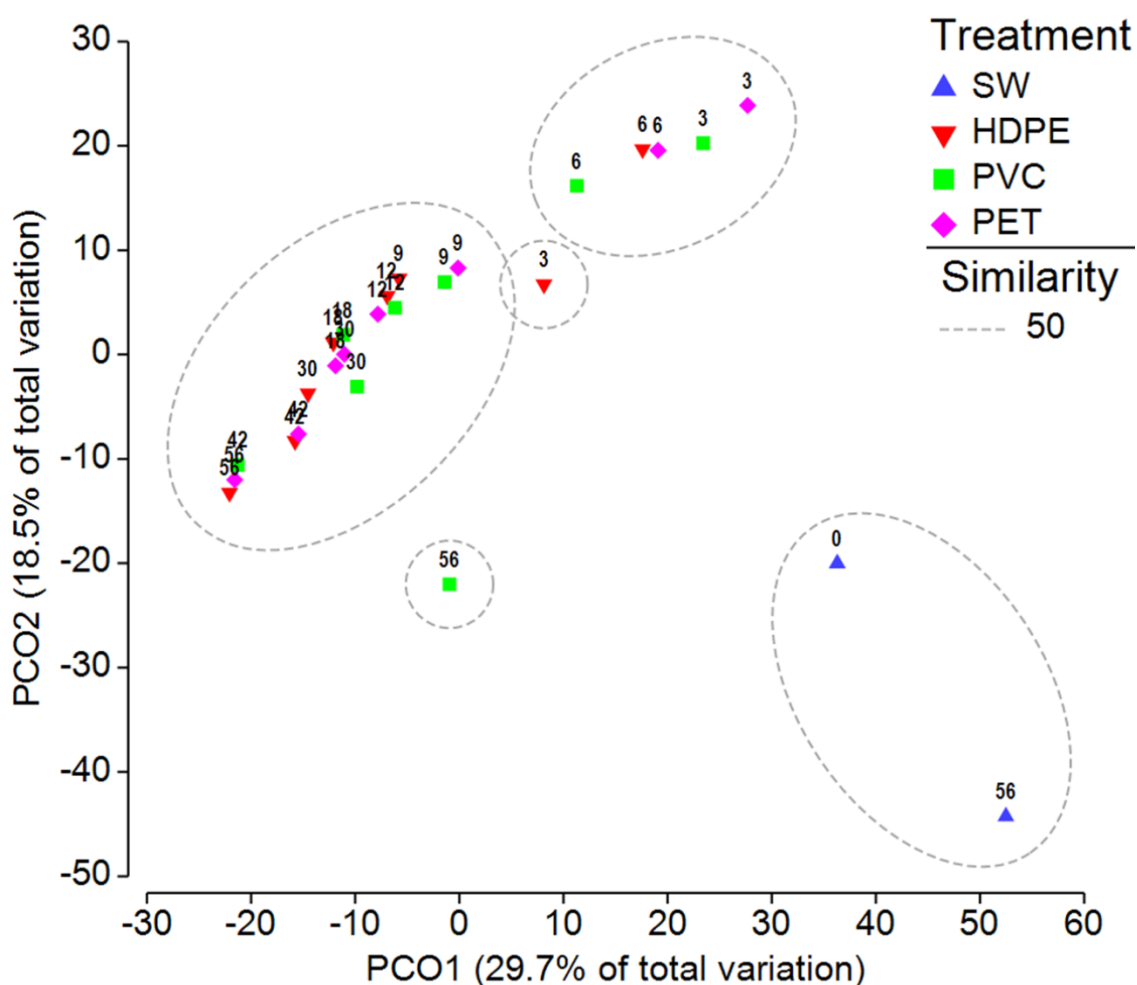


Figure 3.3.7 Principle Coordinate Ordination (PCO) analysis. PCOs representing similarity of the bacterial communities based on Bray-Curtis similarity index, calculated from OTUs ( $>0.1\%$ ) data of the bacterial communities attached on plastics and within seawater samples. The symbols represent the sample types including seawater (SW) and plastics (HDPE, PET and PVC). The numbers indicate the time of sampling in weeks. Clusters represent samples sharing at least 50% similarity of their bacterial communities.

The PCO analysis also revealed that the biofilm communities of all the three plastic types further shifted away from the earlier time points (Figure 3.3.7), indicating that the plastics exposed to seawater displayed a gradual change in bacterial community composition. Furthermore, the community structure of biofilms attached to three different synthetic

plastics were compared to determine the similarity and dissimilarity percentages within each other and with seawater communities. SIMPER analysis showed that bacterial biofilm communities associated with different synthetic polymers differed between 40.9 to 42.9 % from each other, and between 70.3 to 72.0 % from the control seawater communities (Table 3.3.2). The overall similarity within all sampling point for each sample type (SW and plastics) remained between 52.6 to 56.6 % (Table 3.3.2).

Table 3.3.2 SIMPER analysis of bacterial communities, presenting the total similarity within and dissimilarity between the seawater and plastics attached communities. Av.Si%: average percentage similarity within all sampling points, Av.δi %: average dissimilarity between the seawater and plastic attached bacterial communities.

Av.Si%		Av.δi%			
SW	52.63	SW	HDPE	PET	
HDPE	57.32	HDPE	72.00		
PET	56.58	PET	71.04	40.92	
PVC	53.79	PVC	70.31	42.91	42.45

### 3.3.5 The Core Biofilm Communities

To search for sets of OTUs that are shared across all plastic samples at each sampling point, a core microbiome was determined. For this, the OTUs contributing relative abundances of >1% of the total community were detonated as core microorganisms. In total, 36 bacterial core OTUs were identified for all three plastic types (HDPE, PET, PVC). Based on their temporal profile, these core members were classified into three groups: (1) OTUs with higher abundance at the beginning of experiment (early colonisers within 3-12 weeks) e.g. *Ferrimicrobium*, *Pseudahrensia*, *Marinobacter*, *Iamia*, *Candidatus Atelocyanobacterium*, *Cyanobacterium*, *Ilumatobacter*, *Pseudomonas*, *Azospirillum*, *Labrenzia* and *Roseovarius*; (2) OTUs with higher abundance in the middle (intermediary colonisers within 18-30 weeks) of the exposure period, e.g. *Nitrosospira*, *Schlegelella* and (3) OTUs with highest abundance at the end (late colonisers between 42-56 weeks) of the experiment, e.g. *Nitrosospira*, *Arthrobacter*, *Mesorhizobium* (Figure 3.3.8).

Changes in the relative abundance of different bacterial OTUs were observed between different plastics and over time. The Heatmap represents the relative OTU abundance of all 36-core bacterial of all the three-plastic types over the exposure period. (Figure 3.3.8). The early (within 3-12 weeks) biofilm bacterial community on HDPE, PET and PVC were dominated by genus *Ferrimicrobium* (8.5-9.5 %), *Iamia* (3.8-6.5 %), *Pseudahrensia* (4-7.4 %), *Candidatus Atelocyanobacterium* (3.7-4.5 %), *Pseudomonas* and (3.2-4 %). However, the genus *Marinobacter* (14 %) was also present on HDPE in the early stages. In contrast, the genus both *Cyanobacterium* (3.4 % on PET; 6.5 % on PVC) and *Roseovarius* (3.1% on

PET; 2.5 % on PVC) were observed as early colonisers of PET and PVC. The other two OTUs differentiating the PVC biofilm community from other plastic communities were *Anaeromyxobacter* and *Pirellula* with the relative abundance of 3 and 2.3 % respectively. The relative abundances of all these OTUs decreased during the intermediate (18-30 weeks) and late (42-56 weeks) period of exposure.

The plastics biofilm communities showed little changes during the middle of the experiment (18-30 weeks), but the relative abundances of characteristic intermediary colonisers (*Nitrospira* and *Schlegelella*) of all plastic substrates proliferated. In addition, OTUs belonging to the genus *Aciditerrimonas* and *Ilumatobacter* was increased (both approximately 2 %) on the HDPE and PET films during the intermediary period (Figure 3.3.8). Plastics samples taken during the late exposure period (42-56 weeks) showed differences in their attached biofilm community compared to those taken following the early period (3-12 weeks). All plastic types showed enrichment in OTUs affiliated with the genus *Nitrospira* and *Mesorhizobium*. Additionally, increase in the relative abundance of genus *Arthrobacter* (15.6 %), *Frigoribacterium* (2 %) and *Thalassospira* (1 %) was detected on PVC during the late period which was undetectable in other plastic types (Figure 3.3.8).

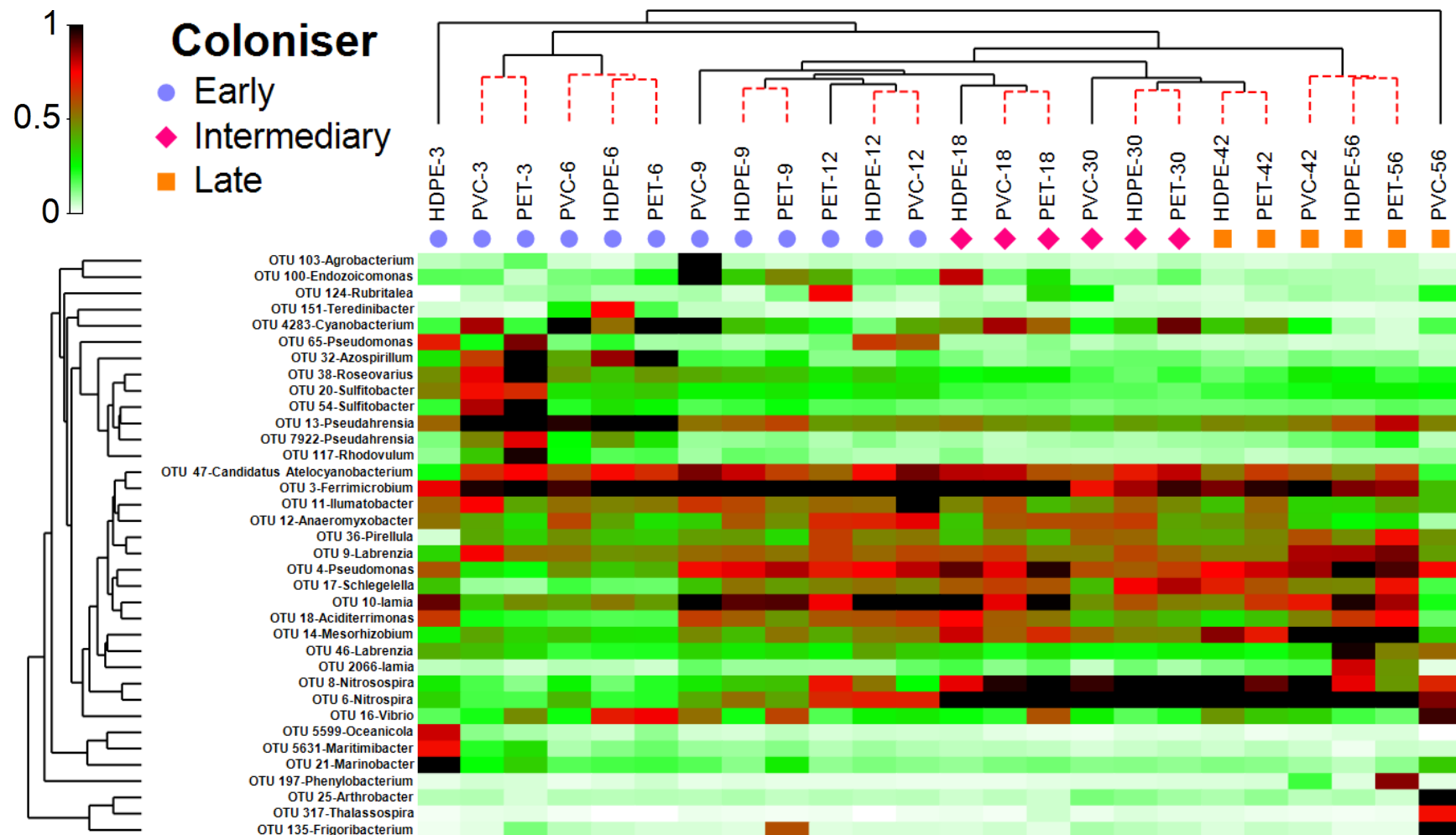


Figure 3.3.8 Heatmap of 36 core OTUs relative abundances across all plastic samples at the beginning indicated as “early” (3-12 weeks), middle indicated as “intermediary” (18-30 weeks) and end indicated as “late” (42-52 weeks) of the exposure. Colour key represents the log-transformed relative abundances (in percentage). Samples were clustered hierarchically based on SIMPROR permutation tests and core OTUs were based on similarity index of association (average linkage). Red lines indicate clusters that are not significantly different ( $p < 0.05$ ).

### 3.4 Discussion

Biofilm development on three chemically distinct synthetic polymers was analysed over a 56-week period, and the characteristic plastic-attached community was studied to assess and identify patterns of biofilm temporal change. After exposing the plastics in the seawater, the formation of a microbial biofilm was observed after the first sampling point (3 weeks of exposure). This is in agreement with previous studies demonstrating that biofilms form quickly (after one week) on plastic surfaces in the marine environments (Lobelle and Cunliffe, 2011, Harrison et al., 2014, De Tender et al., 2017a). Moreover, different progressive stages of biofilm formation were identified based on the increased microbial biomass and a gradual change in the taxonomic composition of biofilm communities over time.

Comparison of the plastic biofilms and the seawater communities demonstrated that both communities were generally distinct regardless of having taxonomic classes in common (Figure 3.3.6). This finding is in agreement with numerous previous studies (Zettler et al., 2013, Oberbeckmann et al., 2014, 2016, Amaral-Zettler et al., 2015, Bryant et al., 2016, De Tender et al., 2015, 2017a, Kirstein et al., 2018) which also observed that the plastic attached biofilm communities were different from free-living seawater communities. This could be due the higher cell density within the attached biofilms as compared to surrounding seawater (Kirstein et al., 2018). A higher cell density supports the formation of complex matrix-stabilized biofilms which comprise of a diversity of microorganisms with vast metabolic capacities (Flemming et al., 2016). Moreover, it promotes strong competition for nutrients in the nutrient-limited marine environments which may force natural selection and also induce certain microorganism to adopt the surface-attached lifestyle or disperse from the biofilm (Karatan and Watnick, 2009, Petrova and Sauer, 2012, Rendueles and Ghigo, 2012, Dang and Lovell, 2016).

OTUs with a relative abundance of  $> 0.1\%$  in each substrate type were analysed and found that amongst those, even if sometimes rare ( $< 0.1\%$ ), all bacterial OTUs were detected on all synthetic polymers. Hence, the dissimilarities in the bacterial community composition observed as a function of the synthetic polymers investigated resulted from variable relative abundance profiles of dominant OTUs. A core group of 36 bacterial OTUs with the relative abundance of  $> 1\%$  were detected across all plastic samples from three weeks onward until the end of the exposure period. Those core organisms were divided into three groups depending on the timing of their peak abundance. Attachment of such core organisms during

the long term biofilm development was also studied by De Tender et al. (2017a) and identified a core group of 25 single OTUs, belonging to the phylum *Proteobacteria*, *Bacteroidetes* and *Verrucomicrobia* on PE. However, it remains unclear whether these “core organisms” are specific for an environment or whether they are also found on other types of plastics, natural surfaces or other hard substrates.

The core community was dominated by genera belonging to phylum *Actinobacteria* (*Ferrimicrobium*, *Iamia*, *Ilumatobacter*, *Arthrobacter*), *Cyanobacteria* (*Candidatus Atelocyanobacterium*), *Proteobacteria* (*Pseudahrensia*, *Marinobacter*, *Pseudomonas*, *Azospirillum*, *Labrenzia*, *Roseovarius*, *Nitrosospira*, *Schlegelella*, *Mesorhizobium* and *Nitrospirae* (*Nitrospira*). A temporal biofilm development study on PE (De Tender et al., 2017a) and 15-month-old mature biofilm study on synthetic plastics in the North Sea (Kirstein et al., 2018) also identified unique core OTUs. Comparison with these studies showed similarities at phylum, class and family level however, no similarities were found at genera level except *Nitrospira*. The dissimilarities between these biofilm communities could be due to the difference in the composition of bacterial taxa of contrasting water bodies (i.e. the North Sea and the English Channel) (De Tender et al., 2015, De Tender et al., 2017a, Kirstein et al., 2018). In addition, it is well established that the environmental factors (e.g. hydrodynamics, temperature, water chemistry and nutrient availability) also influence the composition of biofilm communities (Salta et al., 2013). Thus, indicating that the source communities within surrounding water and the environmental conditions were shaping the colonisation patterns on HDPE, PET and PVC. Similarly, it has also been demonstrated that the plastic associated biofilm communities were distinct with respect to different ocean basin (Amaral-Zettler et al., 2015) and were affected by both seasonal and spatial variations (Oberbeckmann et al., 2014).

In this study, the plastic-attached biofilm communities in this study demonstrated a gradual change in its taxonomic composition throughout the exposure period (Figure 3.3.8). Certain microbial taxa showed dominance during the succession process in addition to the core bacterial community present in the biofilms, but their relative abundances differed over time. The varying relative abundances of these bacterial taxa was pointing towards the different stages of biofilm formation. Therefore, it can be suggested that these specific bacterial taxa are vital for the formation of biofilm and its progression. Previously, members of phylum *Proteobacteria* were reported as primary surface colonisers and the members of phylum *Bacteroidetes* were found to be secondary colonisers of various anthropogenic substrates including plastics in the marine environments (Dang and Lovell, 2000, Dang et al., 2008,

Lee et al., 2008, Elifantz et al., 2013, De Tender et al., 2017a). Although the members of *Proteobacteria* were also observed as early colonisers in the current study however, there were high abundances of some members of *Actinobacteria* initially. This might have occurred because the plastics submerged in the harbour are impacted by intertidal environment having influences of both terrestrial and aquatic environments (Elifantz et al., 2013, De Tender et al., 2015). Moreover, in contrast to previous studies, *Nitrospirae* dominated the late biofilm communities and were therefore suggested as secondary colonisers in this study. A prior study (Kirstein et al., 2018) also reported the high relative abundance of phylum *Nitrospirae* (7–12%) within the 15-month mature biofilms of synthetic plastic biofilms and thus pointing to a possible link to recognise *Nitrospirae* as a secondary coloniser. Moreover, the members of *Nitrospirae* are known for microcolony formation within biofilms after the initial attachment of primary surface colonisers. They can then recruit other microorganisms to the surface through coaggregation (Martiny et al., 2003, Rickard et al., 2003, Martiny et al., 2005, Dang and Lovell, 2016).

Despite the similarities among HDPE, PET and PVC associated bacterial communities, a few distinctively unique OTUs were observed for all the three plastic attached communities across the sampling points. This indicates that physicochemical factors of plastics shape the biofilm communities in addition to the surrounding seawater. These factors include: chemical nature; roughness; vulnerability to weather; surface conditioning and nutrient enrichment (Dang and Lovell, 2016, Frère et al., 2018, Parrish and Fahrenfeld, 2019). OTUs that differentiated HDPE biofilm from both PET and PVC biofilms belonged to the members of *Actinobacteria* (*Aciditerrimonas*) and *Alteromonadales* (*Marinobacter*), both of which were also observed in other plastic biofilms studies (Jiang et al., 2018, Pollet et al., 2018). Members of the *Actinobacteria* and *Alteromonadales* have also been reported for other marine surfaces (Chen et al., 2013, Rajeev et al., 2019). Their ability to produce exopolysaccharides (Lee et al., 2016) and amyloids (Blanco et al., 2012) for providing adherence and resistance to environmental variables respectively, likely make them successful biofilm community members. The unique OTUs of PET attached biofilms belonged to the order *Rhodobacterales* (*Sulfitobacter*), which were previously recognised as dominant surface colonisers in the coastal environment (Dang et al., 2008). *Rhodobacterales* can produce quorum-sensing (a cell to cell communication process) signals which could be involved in several microbial processes such as biofilm formation and its succession process (Gram et al., 2002, Martens et al., 2007). Thus, the presence of these bacteria within the PET biofilm suggest their involvement in shaping biofilm development and its subsequent structuring via intercellular communication and interaction. Moreover, a relatively high



abundance of *Planctomycetales* (*Pirellula*) and *Actinobacteria* (*Arthrobacter* and *Frigoribacterium*) was observed within PVC biofilm communities contrary to HDPE and PET communities. The members of *Planctomycetales* produce holdfast structure to facilitate surface attachment and also colonise marine particles and algal or abiotic surfaces (Egan et al., 2013, Lage and Bondoso, 2014, Bižić-Ionescu et al., 2015).

The biochemical processes within biofilms are typically conducted by a consortia of microorganisms in which a particular process is conducted by the biofilm community as a whole and individual community members contribute specific responsibilities (Dang and Lovell, 2016). Therefore, distinct microbial communities colonising plastics might exhibit various microbial functions within biofilms. One of the notable bacterial taxa within the plastic biofilm assemblages was the genus *Pseudomonas* (Figure 3.3.8). The strains of *Pseudomonas* have been found to have biodegradation potential for HDPE (Balasubramanian et al., 2010), PET (Vague et al., 2019) and PVC (Danko et al., 2004) plastic films. The high abundance of *Pseudomonas* within the plastic biofilms suggests that the plastic may be selecting for bacteria capable of decomposing the plastic compounds. Similarly, the increased abundance of *Cyanobacteria* on all the plastic types was observed compared to surrounding seawater. It has been reported that the prevalence of *Cyanobacteria* on the plastics increased the rates of oxygen production and respiration (Bryant et al., 2016). Assuming this, the occurrence and enrichment of oxygen-producing *Cyanobacteria* on the plastic surface suggest that they could be involved in the oxidation of the polymers.

In some cases, organisms can also enter an obligatory synergistic metabolic association (syntrophic metabolism) in order to perform an otherwise energetically unfavourable metabolic process (Kouzuma et al., 2015). The close association of syntrophic participants within a biofilm facilitates these metabolic reactions. The association of *Nitrosococcus* sp and other ammonia-oxidizing bacteria with nitrite-oxidizing bacteria including *Nitrospira* sp is a notable example of such physiological associations which is commonly encountered within beneficial biofilms in sewage treatment (Juretschko et al., 1998). Here, highly soluble nitrite produced by ammonia oxidation can be quickly used by nitrite oxidizing bacteria, growing in immediate proximity within a biofilm. Such metabolic interactions would not be practical in suspended (i.e. planktonic) populations (Juretschko et al., 1998). The late co-occurrence of *Nitrosospira* (ammonia oxidising bacteria) and *Nitrospira* (nitrate oxidising bacteria) within this studies plastic biofilm communities indicated the prevalence of such an association. This might result in a potential impact on the nitrogen cycle in the areas where plastics accumulate (gyres). Similarly, it has been argued that the presence of nitrogen

fixating bacteria on the plastics creates small nutrient hotspots in otherwise relatively nutrient-poor waters (Bryant et al., 2016).

### **3.5 Conclusions**

This study analysed the temporal biofilm development on distinct synthetic polymers, exposed to seawater. Firstly, it has been demonstrated that biofilms attached to synthetic polymers are significantly different from the surrounding seawater. Secondly, in-depth analysis of bacterial taxa on plastics revealed core microorganisms of the biofilm community which are divided into three groups based on the timing of their highest growth within the plastic biofilms. The results suggest that the members of this bacterial core community are common among all plastic-types. However, some core microbes were substrate-specific despite the occurrence of common bacterial groups on all the substrates, with notable differences observed at genera-level. These microbes could be used as indicator organisms for future studies on plastics within important marine areas (gyers). Furthermore, the role of the core community and their ability to degrade the plastics in the seawater remain unclear because there are fewer studies recognising these microbes as potential degraders. Therefore, further experiments need to be conducted to gain insights into the specific functions and metabolic activities of the core microbial community.

## Chapter 4: Degradation of synthetic plastics in the sea: a long term *in situ* exposure study

### 4.1 Introduction

In Chapter 3, temporal biofilm formation on three chemically distinct synthetic plastics was characterized after long-term exposure in the marine environment. The results showed that the biofilms attached to the plastics were significantly different from the surrounding seawater and a core microbial community was identified. Additionally, the analysis of the core biofilm community revealed the presence of bacteria that could possibly be involved in the degradation of plastics. This, therefore, allows the investigation into whether the biofilm development and prevalence of potential degraders on the polymer surfaces influences the degradation of plastics within the marine waters.

Plastics in marine environments undergo several weathering processes that result in their degradation, this includes: solar irradiation, thermal ageing, oxidation and biofilm growth (Andrady, 2017, Guo and Wang, 2019). Plastic degradation refers to any chemical and physical change that results in the reduction of its average molecular weight and mechanical integrity, and is generally categorised into photodegradation, thermal degradation, hydrolysis, mechanical shearing and biodegradation (Singh and Sharma, 2008, Andrady, 2011, Avio et al., 2017). These degradation processes often lead to fragmentation of macroplastics into microplastics (size < 5 mm) either by abiotic (e.g. sunlight, temperature, moisture, mechanical abrasion) or biotic (microorganisms) factors. Microplastics are well known to negatively impact the marine environment and are reported to be ingested by numerous marine biota (Chapter 1:, Section 1.1.1). The degradation of microplastics changes their physicochemical characteristics, which includes properties related to surface morphology, colour, density and particle size (Zettler et al., 2013, Lambert and Wagner, 2016, Fazey and Ryan, 2016, Zhou et al., 2018, Guo and Wang, 2019). However, degradation processes do not stop at the micro-level, they continue further to produce smaller particles called nanoplastics (Lambert and Wagner, 2016). They have different properties (e.g. increased surface area, higher reactivity) compared to macro and microplastics and are therefore degraded quickly (Klein et al., 2018).

Due to their chemical inertness, plastics are generally resistant to degradation, with estimates for their complete degradation ranging from decades to centuries (Browne et al., 2007). The degradation of plastics has been studied in the marine environments by measuring the change in relevant physical and chemical properties such as: surface morphology; tensile properties; molecular mass distribution; chemical composition and mass loss (Pegram and Andrady, 1989, Lacoste and Carlsson, 1992, Andrady et al., 1993b, O'Brine and Thompson, 2010, Andrady, 2015a, Eich et al., 2015, Cai et al., 2018). These studies focused on early degradation processes to determine the lifetime of the plastics. However, information about the long-term degradation of plastics within the marine environment is very limited, with only a few studies experimenting over periods longer than one year (Brandon et al., 2016, Welden and Cowie, 2017, Biber et al., 2019).

In order to advance our understanding of the fate of plastic debris in the oceans and their ecological impacts on marine organisms, it is important to study the long-term degradation of common synthetic plastics and the variables that govern the degradation process. To date, most of the plastic degradation studies have focused on surface debris. However, the formation of biofilms on marine plastics (Chapter 3:) makes them negatively buoyant (Ye and Andrady, 1991, Fazey and Ryan, 2016, Chubarenko et al., 2016, Guo and Wang, 2019) and results in them sinking into the water column (Andrady, 2011). Moreover, wind-driven mixing results in the submersion of marine plastic debris and it is estimated that approximately 60 % of the debris is submerged in seawater at any given time by this mixing (Kukulka et al., 2012).

Measuring the natural physical and chemical degradation of plastics submerged in seawater will form the basis of this Chapter. This Chapter is the physical sciences companion to Chapter 3:, where long-term biofilm development and dynamics were analysed. The degradation analysis was carried out on chemically distinct synthetic plastics (HDPE, PET and PVC) that were exposed to seawater for 56 weeks in two metres of natural seawater.

The specific aims of this Chapter are:

1. To investigate the long-term degradation of different synthetic polymers, exposed to seawater.
2. To measure changes in the physicochemical properties of plastic including surface morphology, mechanic stability and chemical composition.

## **4.2 Materials and methods**

### **4.2.1 Experimental Design**

In order to determine the degradation of synthetic plastics in seawater, three types of plastic films; high-density polyethylene (HDPE; thickness 0.01 mm; glass transition temperature  $T_g$  -130-100 °C; melting temperature  $T_m$  130-140°C; whitish, semi-opaque) , polyethylene terephthalate (PET; thickness 0.013 mm;  $T_g$  70-80 °C;  $T_m$  245-265°C; colourless and transparent) and polyvinyl chloride (PVC; thickness 0.2 mm;  $T_g$  65-85 °C;  $T_m$  N/A; colourless and transparent) (Goodfellow Cambridge Ltd, Huntingdon, UK) were submerged 2 m deep in the sea at Queen Anne's Battery Plymouth, UK (50°21'57.1"N 4°07'53.8"W) over a 56 week period from 17 July, 2017 to 13 August, 2018. Each plastic material was cut into 40 strips (120 mm × 30 mm) and placed within square frames that were mounted on a supporting beam. Three HOBO Pendant® temperature/light loggers (UA-002-08) (Onset, MA USA) were also attached to the supporting beam to record the daily temperature and light intensity. The plastic samples were collected after 3, 6, 9, 12, 18, 30 42 and 56 weeks of exposure to the seawater. Full details of the sample preparation, deployment in the seawater and collection for subsequent analysis are included in the methods section of Chapter 3:(3.2.1).

### **4.2.2 Removal of Biofilm**

The periodically retrieved plastic samples were covered with biofilm, macrofoulants and sediment particles (Chapter 3:, Section 3.3.1). In order to facilitate the subsequent analyses, the plastic films were washed following a protocol developed by Orr et al. (2004a). For this, each plastic-type was placed in 100 mL capacity conical flask separately with 30 mL of 2 % (w/v) sodium dodecyl sulphate (SDS) solution. The flasks were gently rotated at the speed of 20 rpm for 4 hours to facilitate the detachment of the covering biofilm and other particles. The plastic films were then disinfected with 70 % (v/v) ethanol and washed in distilled water to ensure maximum removal of microbial cell and debris. The washed films were then placed on Whatman® qualitative filter paper (Grade 1) and dried overnight at 40 °C. This procedure was also applied to the control plastics films prior to their analyses.

### **4.2.3 Measuring Plastic Degradation**

#### **4.2.3.1 Plastic Surface Characterisation**

##### **4.2.3.1.1 Scanning Electron Microscopy**

The changes in surface morphology of plastic films were observed through scanning electron microscopy. For this, the dried plastic samples ( $5 \times 5$  mm,  $n = 3$ ) were fixed on 12 mm aluminium stubs using carbon tape and then gold-coated for 40 secs (10 - 12 nm thickness) with an SC7640 Mini Sputter Coater (Quorum Technologies, Laughton, UK) in an argon atmosphere. The plastic surfaces were examined using a Field Emission Gun- Scanning electron Microscope (FEG-SEM) (ZEISS Supra; 55-VP; Zeiss Micro-imaging, Gottingen, Germany), which was operated with an accelerating voltage of 3 kV and a 7 - 10 mm working distance.

##### **4.2.3.1.2 Surface Wettability Analysis**

Changes to surface wettability is an important indicator of plastic degradation (Restrepo-Flórez et al., 2014). Wetting refers to the ability of a liquid to spread out on a solid substrate and maintain contact with the surface (Dwivedi et al., 2017). The surface wettability (the degree of wetting) of the exposed and control plastic films were determined by measuring the contact angle (CA) between a liquid droplet and the films. The surfaces with higher contact angle are considered hydrophobic and those with a lower contact angle are hydrophilic (Webb et al., 2009). Two liquids of varying polarity, Millipore grade distilled water and formamide, were used and their contact angle with plastic films was determined using sessile drop method on an optical tensiometer (Theta Lite, Biolin Scientific, Manchester, UK). In brief, approximately 2  $\mu$ L of liquid was applied on the surface of plastic samples ( $5 \times 5$  mm,  $n= 6$ ) and then images were taken immediately for 20 secs using an attached FireWire digital camera. The contact angle was measured from the captured image at 10 secs, processed in OneAttention software.

#### **4.2.3.2 Mechanical Characterisation**

##### **4.2.3.2.1 Tensile Stress Testing**

Changes in the mechanical properties of plastic films were investigated through tensile stress testing. For this, the plastic films were cut into the shape shown in Figure 4.2.1 using a professional guillotine according to ASTM D882-12 (ASTM-International, 2012). The dimensions of the films after cutting were 25 mm width (b), 100mm total length ( $l$ ) and 50

mm gauge length ( $\ell_o$ ; length used for test). The thickness (h) of each film was measured using an electronic digital micrometre (RS PRO 50-700-001) at every 20 mm along the strip. This produced five reference points for each plastic sample and then the mean thickness was calculated using these readings. The tensile stress at break (the maximum load (N) a material can withstand before it breaks) was measured using a tensile testing machine (Instron 5969, Force capacity 50 kN, Norwood, MA, USA) at the rate of 50 mm min<sup>-1</sup> of crosshead movement.

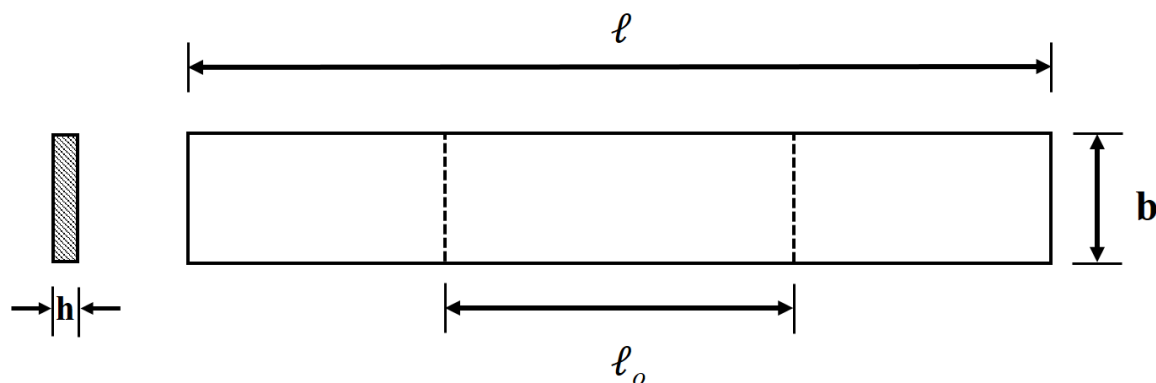


Figure 4.2.1 Schematic illustration of the tensile stress test specimen.  $h$ : mean thickness,  $b$ : width,  $\ell$ : total length,  $\ell_o$ : gauge length.

The maximum tensile stress was calculated using the following Equations Equation 4.2.3.1 and Equation 4.2.3.2.

$$A = bh \quad \text{Equation 4.2.3.1}$$

$$\sigma = \frac{F}{A} \quad \text{Equation 4.2.3.2}$$

In Equation 4.2.3.1,  $A$  is the cross-sectional area which was calculated by multiplying sample width ( $b$ ) and mean thickness ( $h$ ) values. While the tensile stress ( $\sigma$ , expressed in MPa) was calculated by dividing the maximum load ( $F$ ) with the cross-sectional area (Equation 4.2.3.2). The reported values of tensile stress were the average of three replicates per sample. Baseline tensile properties were determined by testing the controls of each type of plastic films.

### 4.2.3.3 Spectroscopic Analysis

#### 4.2.3.3.1 Fourier Transform Infrared (FTIR) Spectroscopy

Fourier transform infrared (FTIR) spectroscopy was conducted in order to determine changes in the molecular composition of plastic films exposed to seawater. It is an efficient and non-destructive technique used for the identification of chemical functional groups based on their well-known infrared absorption bands (Skoog et al., 2017). There are two main operating modes for FTIR spectroscopy, transmittance and reflectance. Transmittance mode is limited to characterising transparent and thin material while the non-transparent and other solid samples can be characterised in reflectance mode (Ojeda et al., 2009). In addition, there is another reflective method of operation known as attenuated total reflectance FTIR (ATR-FTIR) spectroscopy (Skoog et al., 2017). In this method, the infrared rays are passed through a highly refractive crystal which is in contact with the sample surface. This results in a spectrum with high signal-to-noise ratios and minimal distortion (Bokria and Schlick, 2002, Li et al., 2007).

In this study, FTIR analysis of plastic films was performed using attenuated total reflectance FTIR (ATR-FTIR) spectroscopy. For this, an FTIR (Nexus, Thermo Nicolet, Madison, USA) spectrophotometer coupled with Specac Golden Gate ATR device was used. The ATR accessory consisted of a diamond crystal at an incidence angle of 45 degrees. The ATR diamond was cleaned with 70 % ethanol before performing the analysis and a background scan was performed between each sample. The cleaned plastic films ( $5 \times 5$  mm,  $n = 6$ , each plastic type at each sampling point) were compressed against the ATR crystal to ensure good contact between the sample and the crystal. Spectra were collected in reflectance mode within the range of  $400\text{--}4000\text{ cm}^{-1}$  wavenumber using a spectral resolution of  $2\text{ cm}^{-1}$ . Each spectrum was collected using 100 co-added scans to increase the signal-to-noise ratio in OMNIC software (v. 9.2).

The carbonyl bond area within the infrared spectra was measured as a proxy for degradation and change based on previous research (Socrates, 2004, Pavia et al., 2008). The infrared (IR) signal of carbonyl bonds appear in the wide range of wavelengths (e.g.  $1550\text{--}1810\text{ cm}^{-1}$ ) (Socrates, 2004, Brandon et al., 2016) and the peak at  $1715\text{ cm}^{-1}$  (ketone absorption band) is commonly used as the central reference for the carbonyl bond absorption region (Pavia et al., 2008). The carbonyl bond peaks within this region are indicative of chemical change and appear due to the oxidation of carbon within the plastic hydrocarbon chains (Brandon et al.,



2016). The extent of this chemical oxidation is often quantified as according to the carbonyl index (CI), using the following Equation 4.2.3.3.

$$\text{Carbonyl Index (CI)} = \frac{A_{C=O}}{A_{\text{Reference Peak}}} \quad \text{Equation 4.2.3.3}$$

Where  $A_{C=O}$  is the absorbance of  $C=O$  stretching area ( $1550\text{-}1810\text{ cm}^{-1}$ ) and the  $A_{\text{Reference peak}}$  is the absorbance of reference peak area which is considered to be not changed by the degradation processes and varies in terms of its location within IR spectra of the material of interest (Socrates, 2004). The area of reference peaks used for this study is shown in Table 4.2.1.

Table 4.2.1 Wavenumbers selected as reference peak area for HDPE, PET and PVC to measure the carbonyl index.

Plastics	Area of reference peaks	References
HDPE	$2908\text{-}2920\text{ cm}^{-1}$	(Nishikida and Coates, 2003, Socrates, 2004)
PET	$1395\text{-}1415\text{ cm}^{-1}$	(Noda et al., 2007, Asensio et al., 2009, Nguyen-Tri and Prud'homme, 2019)
PVC	$1422\text{-}1438\text{ cm}^{-1}$	(Beltran and Marcilla, 1997, Noda et al., 2007)

#### 4.2.4 Statistics

The quantitative contact angle, tensile stress and carbonyl index data was compared against two factors; plastic types (HDPE, PET, PVC) and exposure period (0, 3, 6, 9, 12, 18, 30, 42, 56 weeks) using mixed factorial repeated-measures ANOVA. A general linear model (GLM) with two-way interactions (plastic-type  $\times$  time (weeks)) was used to analyse within-subject (time) and between-subject (plastic-type) difference during the experiment. Posthoc Tukey tests were used when non-significant interactions were observed, to identify the significant effects. These statistical analyses were conducted using IBM SPSS Statistics for Windows v. 22.0.

### 4.3 Results

#### 4.3.1 Temperature and Light

Temperature and light levels of the exposure site were measured using loggers attached to the sample supporting beam. The average monthly seawater temperature was  $12.75\text{ }^{\circ}\text{C}$  over

the course of the exposure experiment. The seawater temperature varied between 7.83 °C, recorded in March, and 17.15 °C, recorded in July, with clear seasonal variations (Figure 4.3.1). The average monthly light intensity was 35.5 Lux during the exposure period. The highest monthly light intensity of 122.5 Lux was recorded for July 2017 and the lowest intensity took place in the month of November 2017 (0.46 Lux) (Figure 4.3.1).

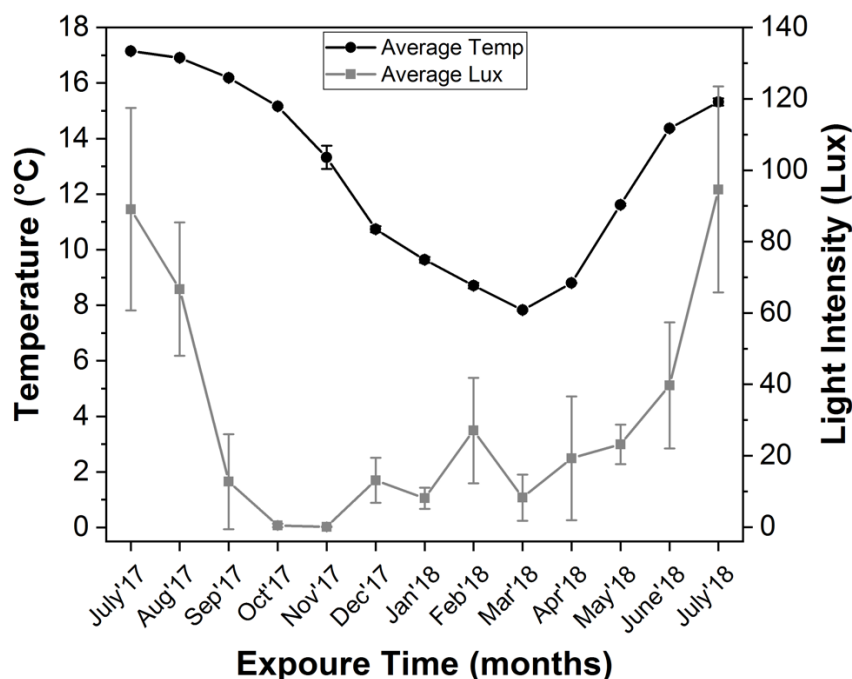


Figure 4.3.1 Average monthly temperature (°C) and light intensity (lux) over the period of exposure experiment. The error bars indicate standard error of the mean.

#### 4.3.2 Morphological Changes

Scanning electron microscopy was used to examine the physical surface topography of the three plastic types (HDPE, PET and PVC) at each sampling point. SEM analysis showed diverse alterations in the surface properties of all plastic types within an exposure time of 56 weeks in the seawater. The examination of the control HDPE film revealed a smooth and featureless surface (Figure 4.3.2 a). Similarly, HDPE films remained largely homogenous, with no visible features after 3 and 6 weeks in the seawater (Figure 4.3.2 b, c). After 6 weeks, small fissures appeared on the HDPE film (Figure 4.3.2 d) and were more visible after 9 and 12 weeks of exposure time along with small holes (Figure 4.3.2 e, f). SEM analysis of the HDPE films revealed that surface features including microcracks and grooves became more apparent between 30 and 42 weeks of incubation (Figure 4.3.2 g, h). The topography of HDPE exhibited large cracks, the remnants of the biofilm and the areas where the upper surface layer was separating from the underneath layer, following 56 weeks (Figure 4.3.2 i).

Scanning electron images of PET films, obtained before the seawater exposure (control), showed a uniform smooth surface (Figure 4.3.3 a). Similarly, the surface of PET films did not show notable changes and presented uniformity after sampling at 3, 6, 9, 12 18 weeks of exposure. The SEM images of these samples bore a striking resemblance to the control films (Figure 4.3.3 b-f). However, the surface morphology after 30 weeks of immersion in seawater showed the development of small cracks on PET films (Figure 4.3.3 g). The cracks were more noticeable on the PET films, collected after 42 and 56 weeks of incubation (Figure 4.3.3 h, i).

Inspection of the PVC control films revealed a relatively uniform surface with few spheroid shape granules on the surface (Figure 4.3.4 a). Following 3 and 6 weeks of submersion in seawater, the PVC films did not show any signs of surface alterations and remained largely similar to the control surface (Figure 4.3.4 b, c). However, the PVC films collected after 9 weeks, appeared to have an uneven surface containing numerous small pits (Figure 4.3.4 d). These pits were more apparent at 12, 18 and 30 weeks and presented a hollowed-out appearance (Figure 4.3.4 e-g). Similarly, the SEM images showed a highly irregular surface with numerous pits at the end of incubation (42 and 56-week) along with adhering material, probably the biofilm particles remained on the surface due to incomplete removal (Figure 4.3.4 h, i).

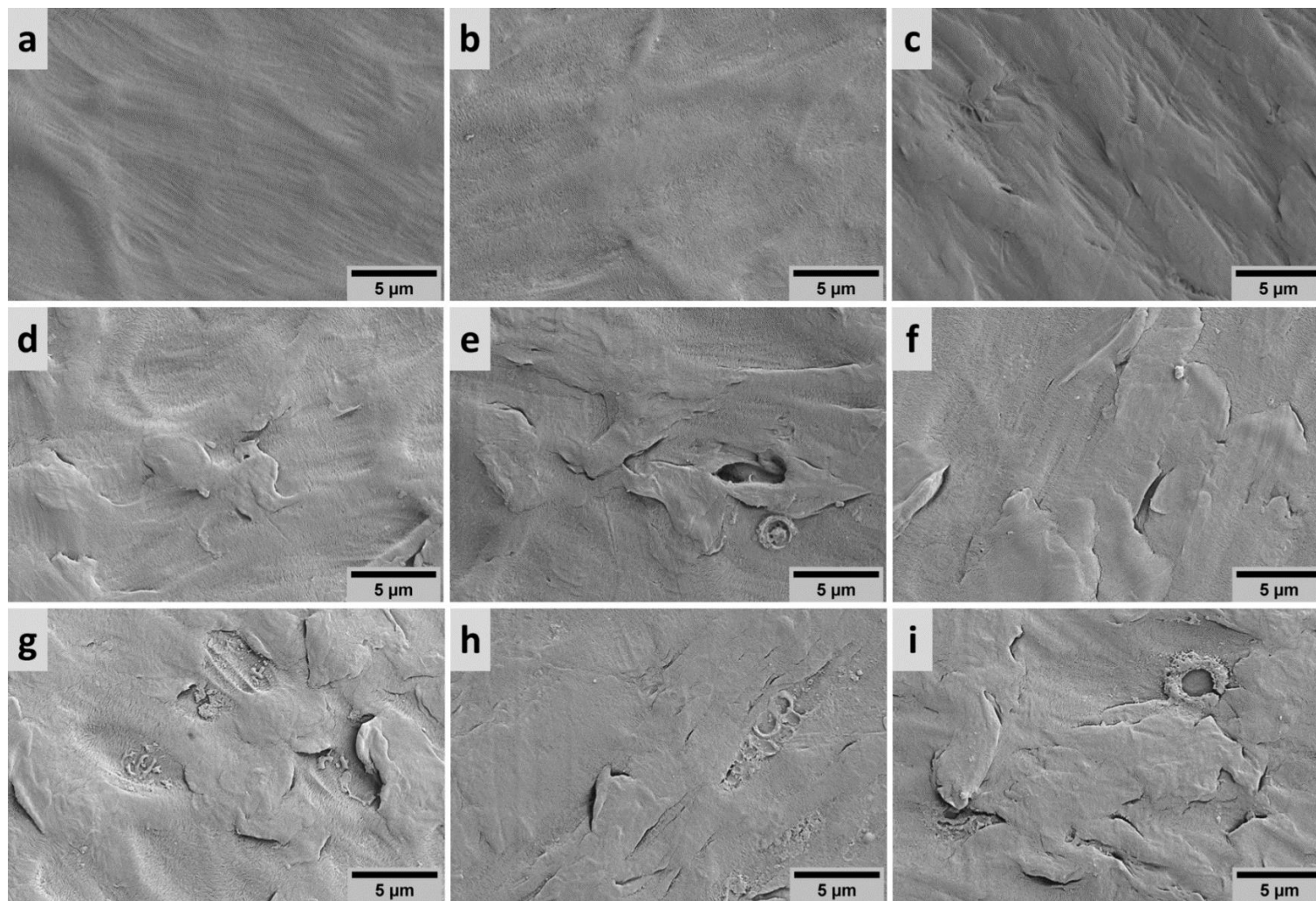


Figure 4.3.2 Scanning electron micrographs showing the topography of HDPE films, sampled after (a) control, (b) 3, (c) 6, (d) 9, (e) 12, (f) 18, (g) 30, (h) 42, (i) 56 -week exposure in sea water. Scale bar = 5 µm.

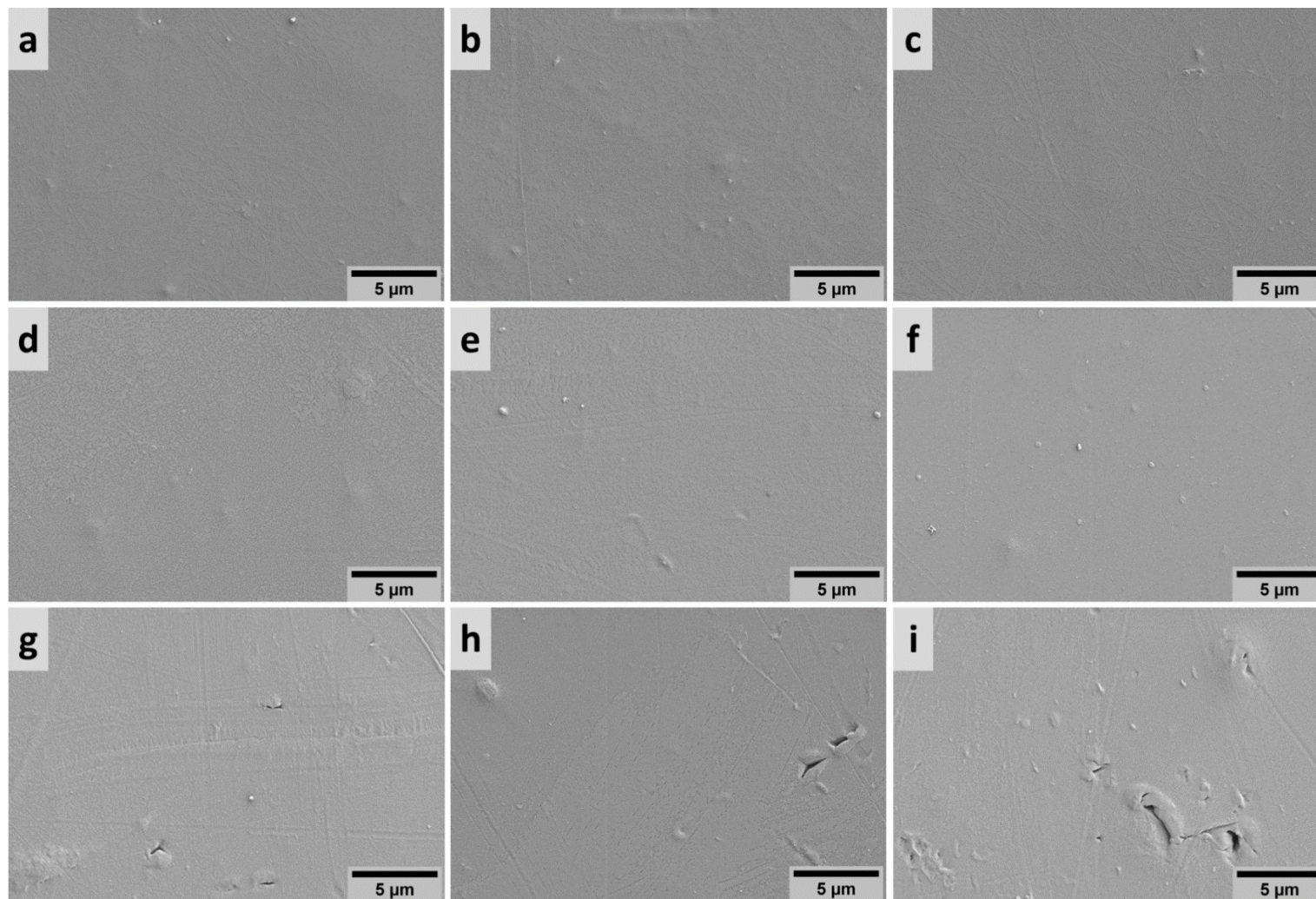


Figure 4.3.3 Scanning electron micrographs showing the topography of PET films, sampled after (a) control, (b) 3, (c) 6, (d) 9, (e) 12, (f) 18, (g) 30, (h) 42, (i) 56 -week exposure in sea water. Scale bar = 5  $\mu\text{m}$ .



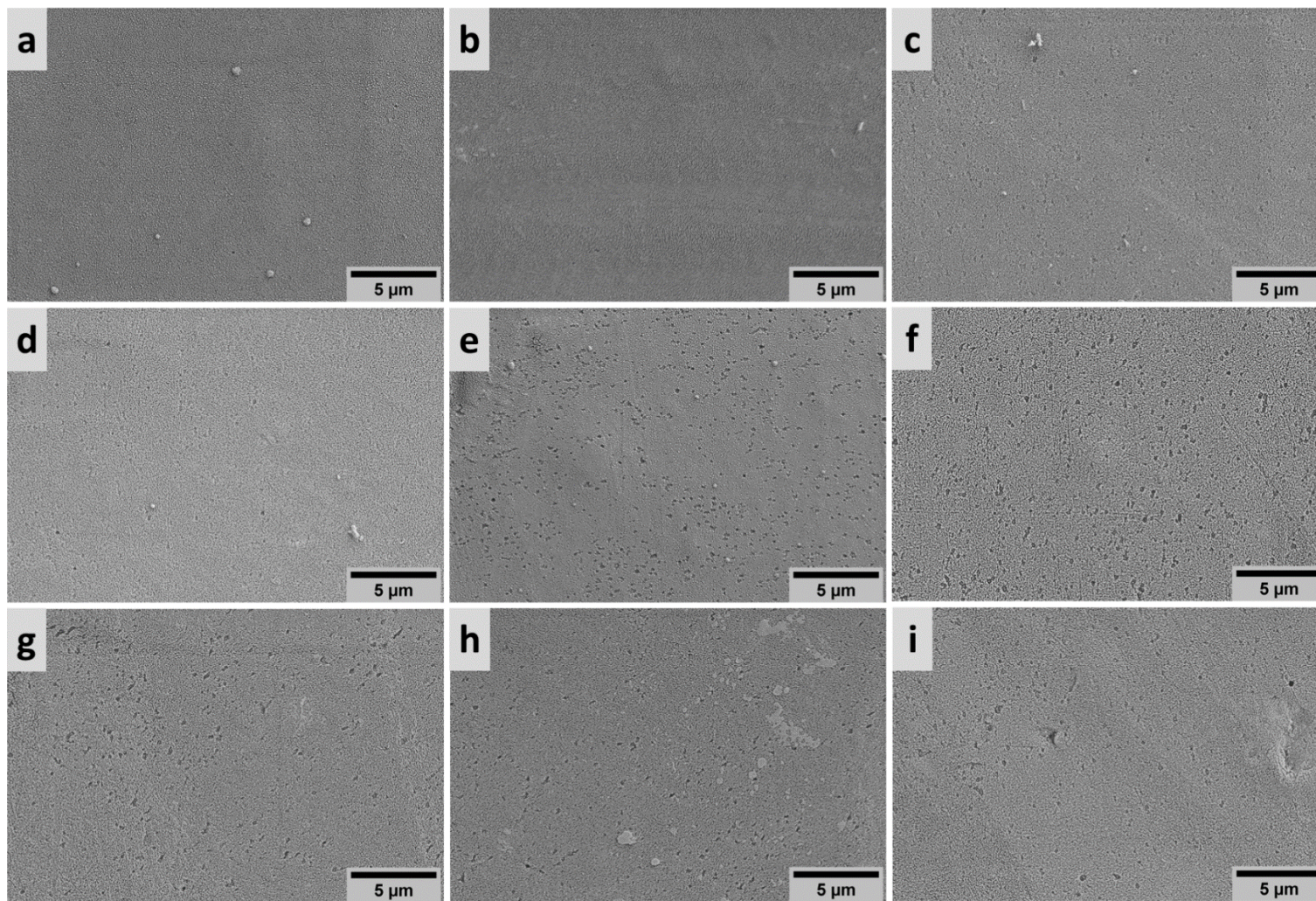


Figure 4.3.4 Scanning electron micrographs showing the topography of PVC films, sampled after (a) control, (b) 3, (c) 6, (d) 9, (e) 12, (f) 18, (g) 30, (h) 42, (i) 56 -week exposure in sea water. Scale bar = 5  $\mu\text{m}$ .

### 4.3.3 Contact Angle Measurement

The contact angle (CA) was measured to analyse the changes in surface wettability of plastics. The water contact angle of untreated (control) HDPE, PET and PVC films were  $87.57^\circ \pm 4.37^\circ$ ,  $74.55^\circ \pm 2.38^\circ$  and  $85.69^\circ \pm 3.04^\circ$  respectively, indicating that the surfaces of all plastic-types were initially hydrophobic due to a high water contact angle (Figure 4.3.5 a). After the submersion of all plastics in the seawater, the water CA decreased gradually over 56 weeks (Figure 4.3.5 a). The CA (H<sub>2</sub>O) of HDPE films decreased significantly (HDPE, F (8, 45) 3.14, p = 0.007 Table 4.3.1) from  $85.85^\circ \pm 2.67^\circ$  (after 3 weeks) to  $78.37^\circ \pm 2.91^\circ$  at the end of the experiment. Similarly, a significant (PVC, F (8, 45) 3.62, p = 0.002 Table 4.3.1) gradual decrease in the water CA of PVC films was also measured. The CA (H<sub>2</sub>O) of PVC films was decreased from  $82.86^\circ \pm 2.55^\circ$  (3 weeks) to  $76.12^\circ \pm 5.56^\circ$  after 56 weeks. In contrast to HDPE and PVC, the water CA of PET films was slightly decreased from  $72.82^\circ \pm 3.31^\circ$  to  $70.22^\circ \pm 3.51^\circ$  over the exposure period (Figure 4.3.5 a).

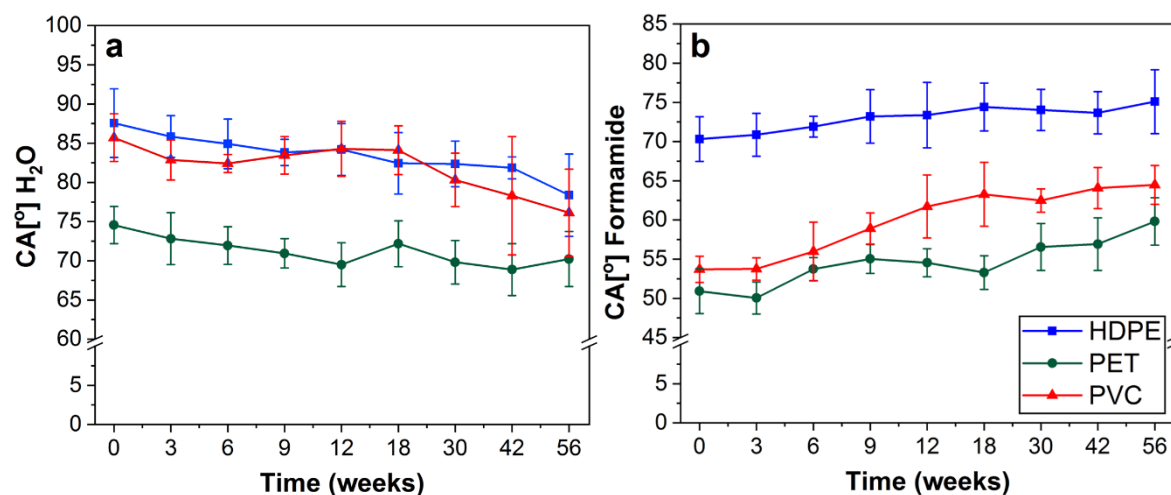


Figure 4.3.5 Contact angle (CA) measurement for HDPE, PET and PVC films as a function of sampling time. (a) water contact angle; (b) formamide contact angle. The error bars indicate the standard error.

Further insights into the surface properties of the plastics were determined by measuring the formamide contact angle. The contact angle of formamide droplet resting on the untreated surface of HDPE ( $70.32^\circ \pm 2.84^\circ$ ), PET ( $50.92^\circ \pm 2.85^\circ$ ) and PVC ( $53.69^\circ \pm 1.67^\circ$ ) were lower compared to water CA, i.e. they spread more across the plastic surfaces. Notably, the formamide contact values increased for all plastic surfaces after the submersion in the seawater (Figure 4.3.5 b). The highest increase was observed in the recorded for PET and PVC films. For PET, it increased significantly (PET, F(8, 45) 8.27, p < 0.001 Table 4.3.1) from  $50.05^\circ \pm 2.04^\circ$  (after 3 weeks) to  $59.82^\circ \pm 3.03^\circ$  (after 56 weeks) while the contact value for PVC increased significantly (PVC, F(8, 45) 14.54, p < 0.001 Table 4.3.1) from the first sampling (3 weeks) value of  $53.75^\circ \pm 1.42^\circ$  to  $64.47^\circ \pm 2.27^\circ$  at the end of

incubation. Similarly, the measured values for formamide contact angle were also increased slightly for HDPE films (from  $70.87^\circ \pm 2.74^\circ$  to  $75.09^\circ \pm 4.07^\circ$  ), however, this increase was negligible and repeated measure ANOVA test showed no significant difference within the sampling points (HDPE,  $F(8, 45) 1.96, p = 0.07$  Table 4.3.1).

*Table 4.3.1 Mixed factorial repeated measures ANOVA results of H<sub>2</sub>O and formamide contact angle (CA) data in relation to plastic-type and exposure time.*

CA-H <sub>2</sub> O					
Source	SS	df	Mean Square	F	p
HDPE	281.39	8	35.17	3.14	0.007
PET	154.76	8	19.34	2.34	0.034
PVC	465.38	8	58.17	3.62	0.002
Plastic-type	4900.24	2	2450.12	131.74	< 0.001
Time (weeks)	713.63	8	172.4	8.12	< 0.001
Plastic-type × Time (weeks)	187.90	16	22.69	1.07	0.39
CA-Formamide					
Source	SS	df	Mean Square	F	p
HDPE	126.02	8	15.75	1.96	0.07
PET	396.06	8	49.51	8.27	< 0.001
PVC	913.83	8	114.23	14.54	< 0.001
Plastic-type	9818.15	2	4909.07	1018.69	< 0.001
Time (weeks)	1160.43	8	145.04	19.08	< 0.001
Plastic-type × Time (weeks)	275.60	16	17.23	2.27	0.006

#### 4.3.4 Changes in Tensile Properties

Tensile stress tests were performed to determine the changes in the mechanical properties of all three plastic types. At first, the maximum tensile stress of all unexposed plastic films (negative controls) was measured. The tensile stress of control PET films was higher than the PVC ( $164.1 \pm 22.4$  and  $55.7 \pm 4.2$  MPa, respectively) while the HDPE films had the lowest tensile stress ( $28.6 \pm 0.3$  MPa). After exposing to seawater, all plastic-types were recovered successfully at each sampling point and analysed visually. No surface area loss was measured, and all samples remained intact in the seawater. However, the maximum tensile stress of all the plastic-types (HDPE, PET and PVC) decreased in seawater over the exposure period, indicating some degree of degradation (Figure 4.3.6). Moreover, the rate at which the tensile stress decreased, was different for all plastic-types.



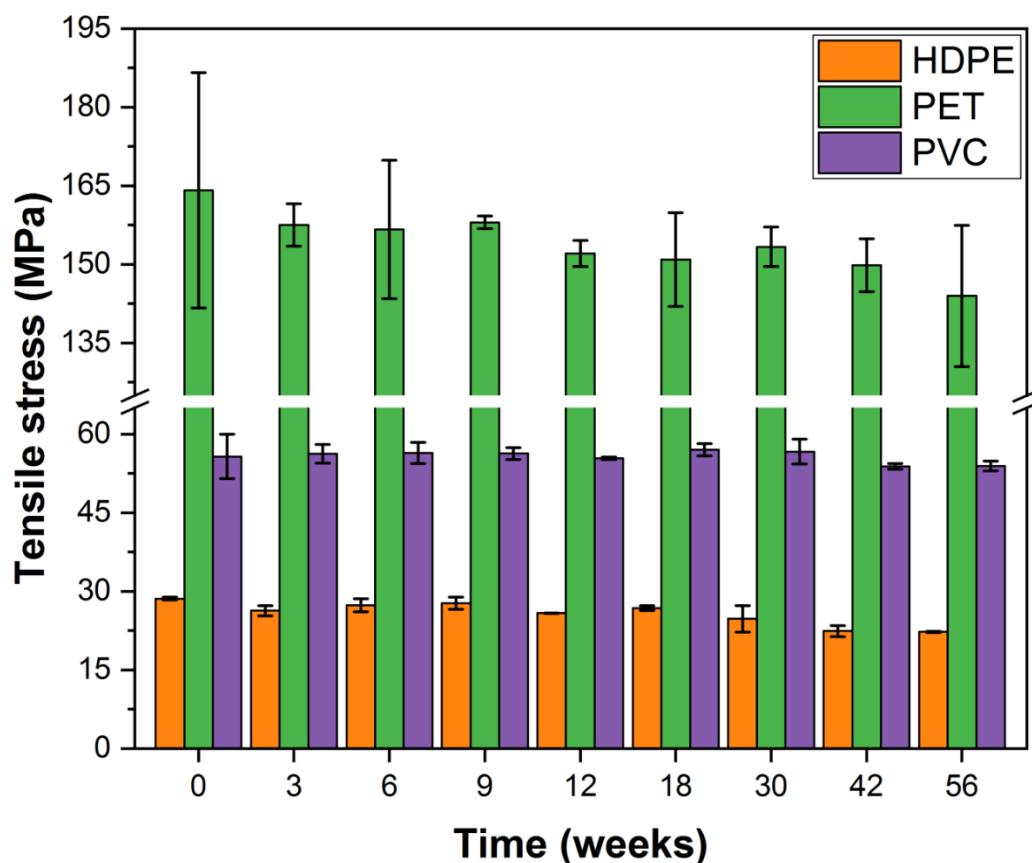


Figure 4.3.6 Tensile stress (MPa) of HDPE, PET and PVC films, exposed to seawater over the period of 56 weeks. The error bars indicate the standard error.

The tensile stress results obtained for PET and PVC films were similar as shown in (Figure 4.3.6). More specifically, the tensile stress of both of these plastic films decreased over time but did not show a significant within the sampling points (repeated measure ANOVA: PET,  $F_{(8,18)} 101.12, p = 0.47$  and PVC,  $F_{(8,18)} 3.95, p = 0.46$ , Table 4.3.2). Similarly, the maximum tensile stress of HDPE also decreased over time from  $26.31 \pm 0.97$  (after 3 weeks) to  $22.2 \pm 0.1$  MPa (after 56 weeks). However, in contrast to PET and PVC, there was a significant loss of tensile stress of the HDPE samples (repeated measure ANOVA: HDPE,  $F_{(8,18)} 11.8, p < 0.001$ , Table 4.3.2).

Table 4.3.2 Mixed factorial repeated measures ANOVA results of tensile stress data in relation to the plastic-type and exposure time.

Source	SS	df	Mean Square	F	p
HDPE	120.81	8	15.100	11.778	< 0.001
PET	808.99	8	101.123	.997	0.47
PVC	31.618	8	3.952	1.021	0.46
Plastic type	243173	2	232532.42	3145.61	< 0.001
Time (weeks)	585.99	8	73.25	2.5	0.05
Plastic type $\times$ Time (weeks)	375.41	16	44.87	0.61	0.77

### 4.3.5 Changes in ATR-FTIR Spectra

The attenuated reflectance (ATR) Fourier transform infrared (FTIR) spectroscopy was used to analyse the chemical changes on the surface of plastics within the experiment. The ATR-FTIR spectra of all three plastic types (HDPE, PET and PVC) at each sampling point were plotted in a comparative way in Figure 4.3.7, Figure 4.3.8 and Figure 4.3.9 respectively. For HDPE, six characteristic peaks were identified at wavenumbers of 717, 730, 1462, 1472, 2846 and 2915  $\text{cm}^{-1}$  (Figure 4.3.7 a). The large intensity peaks at 717 and 730  $\text{cm}^{-1}$  correspond to the rocking vibration of  $\text{CH}_2$  groups and the crystallinity of HDPE. The medium intensity peaks at 1462 and 1472  $\text{cm}^{-1}$  are attributed to  $\text{CH}_2$  bending and scissoring groups and represent crystallinity. The strong sharp peaks at 2847 and 2915  $\text{cm}^{-1}$  correspond to  $\text{CH}_2$  groups and asymmetric and symmetric C–H stretching respectively (Figure 4.3.7 a). After being exposed to the seawater, no new peaks appeared for HDPE during the exposure period. However, the intensity of existing characteristics peaks varied at different sampling intervals, which can be attributed to degradation (Gulmine et al., 2003, Da Costa et al., 2018). There was a general increase in the peaks of 717, 730, 1462, 1472, 2846 and 2915  $\text{cm}^{-1}$  at 9 weeks (Figure 4.3.7 b-e), then a decrease after 12 weeks that lasted until 42 weeks, where it began to increase again at 56 weeks. The increase in the peak intensity at 2915  $\text{cm}^{-1}$  indicated the decrease in the oxidation of the polymer chain (Figure 4.3.7 d). To quantify the extent of oxidation during the exposure period, the carbonyl index of HDPE was calculated. A nonlinear change in the carbonyl index was observed, with a decrease from 0 to 9 weeks, a gradual increase from 12 to 42 weeks and another decrease at the end of the exposure period (at 56 weeks) (Figure 4.3.10 a).

The ATR-FTIR spectra of PET had six characteristic peaks at the wavenumbers of 722, 871, 1094, 1241, 1410 and 1712  $\text{cm}^{-1}$  (Figure 4.3.8 a). The peak at 722  $\text{cm}^{-1}$  corresponds to C–H bending vibrations of the benzene rings and the weak peak at 871  $\text{cm}^{-1}$  represent the C–C out of plane bending bond. The intense peaks at 1094 and 1241  $\text{cm}^{-1}$  correspond to ether aliphatic (C–O stretching) bonds and the weak peak C=C stretching appears at 1410  $\text{cm}^{-1}$ . The strong band at 1712  $\text{cm}^{-1}$  is attributed to ester (carbonyl C=O stretching) (Figure 4.3.8 a). When exposed to seawater, the intensities of the PET characteristic peaks at 1241 and 1712  $\text{cm}^{-1}$  were changed. In the beginning, there was no change in the intensities of these peaks (from 0 to 18 weeks) but decreased at 30 weeks. Then peak intensities began to increase again after 42 weeks until 56 weeks (Figure 4.3.8 d,e). The ATR-FTIR spectra of seawater exposed PET also demonstrated new peaks, which were not typical for PET. These peaks were observed gradually during the 56 weeks incubation period at the following

positions: (a) at  $970\text{ cm}^{-1}$  (Figure 4.3.8 b) (b) at  $1120\text{ cm}^{-1}$  (Figure 4.3.8 c) and (c) at  $1578\text{ cm}^{-1}$  (Figure 4.3.8 e), corresponding to C–H out of plane bend, C–O stretching, and ring C=C stretching respectively. Furthermore, the carbonyl indices for PET at each sampling interval were determined (Figure 4.3.10 b) relative to the C=C stretching peak at  $1410\text{ cm}^{-1}$ . There was no change in the carbonyl indices of PET initially (from 0 to 9 weeks) except for a transient dip at 12 weeks. It began to increase after 18 weeks of exposure, indicating an increase in the oxidation of PET which lasted until 30 weeks, then decreased at 42 weeks. However, another increase in the carbonyl index of PET was observed towards the end of the experiment (at 56 weeks) (Figure 4.3.10 b).

For PVC, the characteristic peaks were observed at the wavenumbers of 610, 1240, 1331, 1427, 2910 and  $2970\text{ cm}^{-1}$  (Figure 4.3.9 a). The strong peak at  $610\text{ cm}^{-1}$  corresponds to C–Cl bond while the peaks at 1240 and  $1331\text{ cm}^{-1}$  are assigned to bending bond of C–H near Cl. The weak band at  $1427\text{ cm}^{-1}$  and the shoulder peak at  $1434\text{ cm}^{-1}$  is attributed to C–H aliphatic bending bond. Moreover, the peaks at 2910 and  $2970\text{ cm}^{-1}$  represent the asymmetric vibrations of C–H stretching (Figure 4.3.9 a). Among the native peaks of PVC, the intensity of  $610\text{ cm}^{-1}$  decreased and a wide band was observed after 56 weeks incubation in the seawater (Figure 4.3.9 b). Similarly, at  $965\text{ cm}^{-1}$  (correspond to the vibrational bands of the rocking of  $\text{CH}_2$  groups) and  $1434\text{ cm}^{-1}$  (C–H aliphatic bending bond peak), there was a decrease and the peaks broadened (Figure 4.3.9 c, d). Apart from the characteristic peaks, a small band at  $1735\text{ cm}^{-1}$ , attributed to C=O bond, was observable in the spectra of PVC at each sampling point. The intensity of this peak remained unaltered initially (from 0 to 12), then gradually decreased slightly from 18 weeks to 42 weeks but the maximum decrease in  $1735\text{ cm}^{-1}$  band was observed after 56 weeks of exposure (Figure 4.3.9 d). Moreover, the carbonyl indices for PVC after seawater exposure were determined (Figure 4.3.10 c) relative to the C–H peak at  $1427\text{ cm}^{-1}$ . No obvious trends, however, were observed in the calculated carbonyl indices (Figure 4.3.10 c).

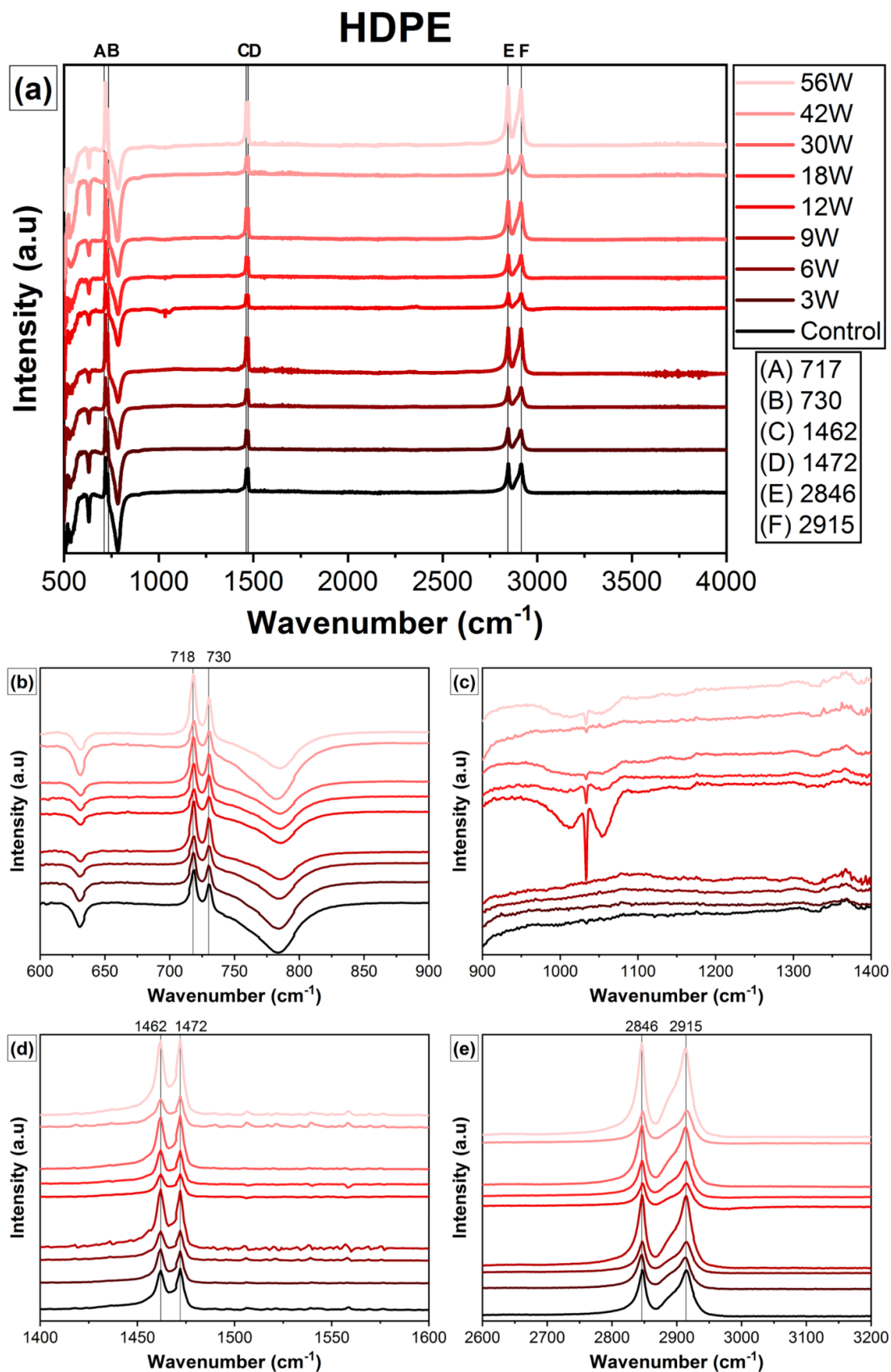


Figure 4.3.7 ATR-FTIR spectra of HDPE films. (a) Comparative spectra of HDPE outer surface at 0 (control), 3, 6, 9, 12, 18, 30, 42, and 56 -weeks of exposure to seawater, with the intensity of peaks recorded as arbitrary units (a.u.). For better visualisation, the ATR-FTIR spectra are enlarged at wavenumbers (b) 600-900, (c) 900-1400, (d) 1400-1600 and (e) 2600-3200.

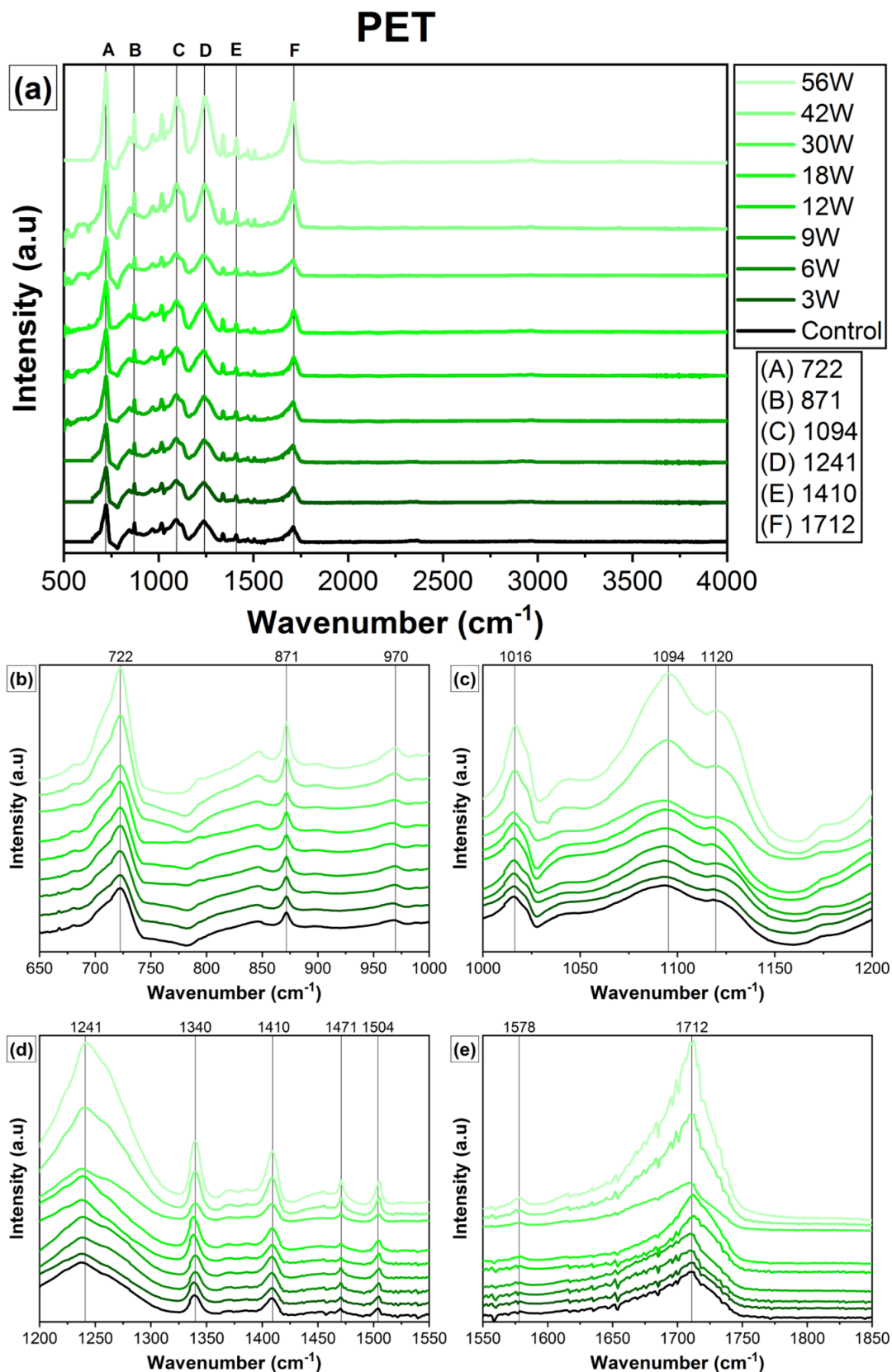


Figure 4.3.8 ATR-FTIR spectra of PET films. (a) Comparative spectra of PET outer surface at 0 (control), 3, 6, 9, 12, 18, 30, 42, and 56 -weeks of exposure to seawater, with the intensity of peaks recorded as arbitrary units (a.u.). For better visualisation, the ATR-FTIR spectra are enlarged at wavenumbers (b) 650-1000, (c) 1000-1200, (d) 1200-1550 and (e) 1550-1850.

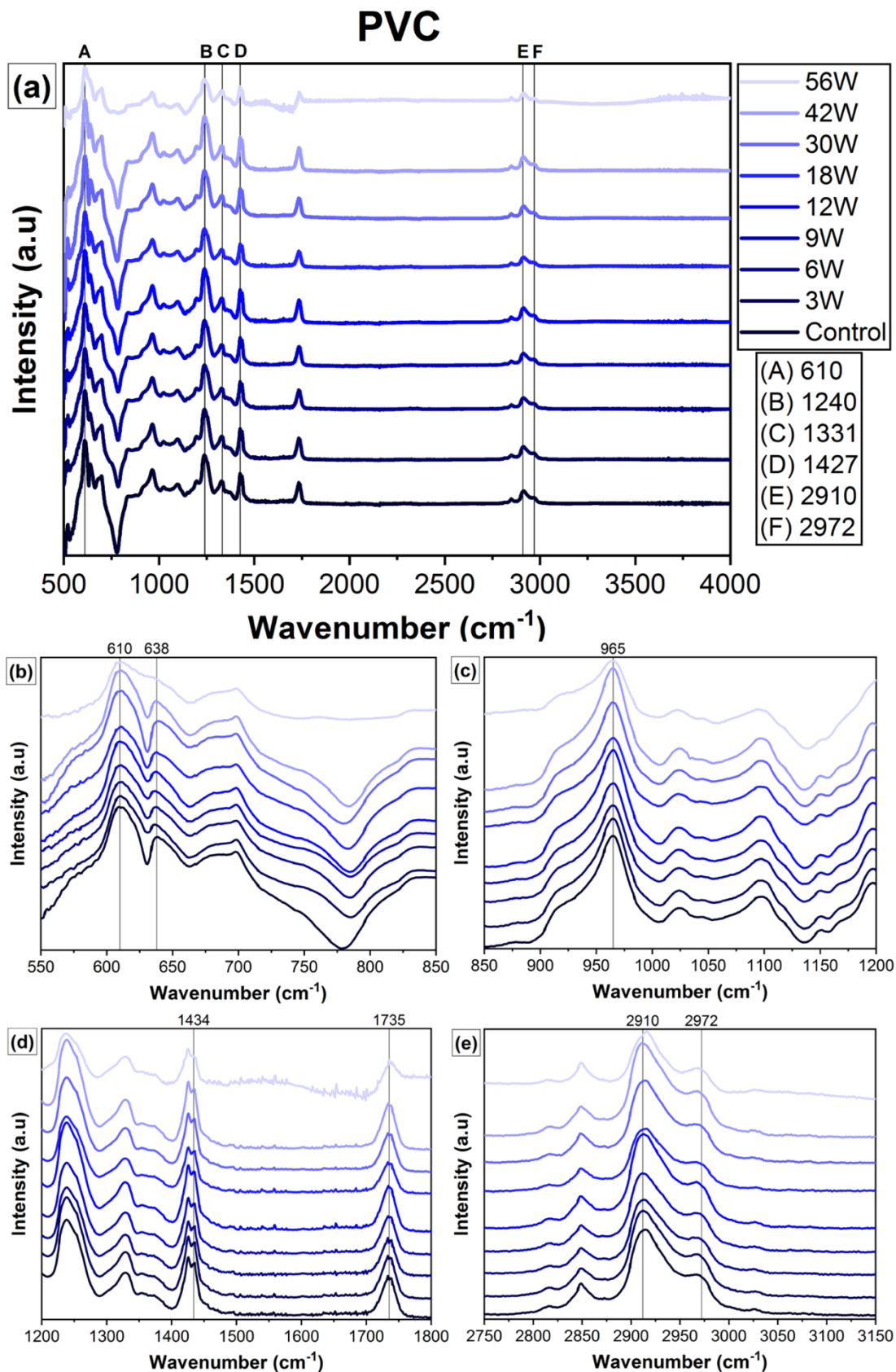


Figure 4.3.9 ATR-FTIR spectra of PVC films. (a) Comparative spectra of PVC outer surface at 0 (control), 3, 6, 9, 12, 18, 30, 42, and 56 -weeks of exposure to seawater, with the intensity of peaks recorded as arbitrary units (a.u.). For better visualisation, the ATR-FTIR spectra are enlarged at wavenumbers (b) 550-850, (c) 850-1200, (d) 1200-1800 and (e) 2750-3150.

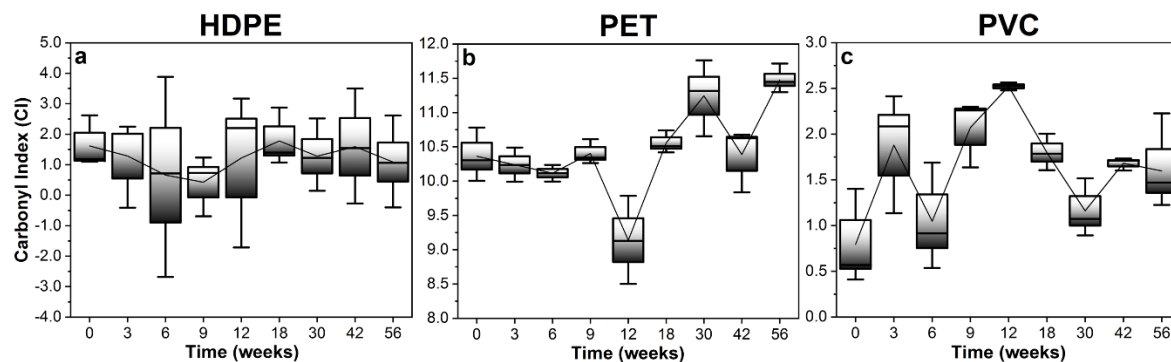


Figure 4.3.10 Boxplots representing the carbonyl index (CI) measurements for HDPE, PET and PVC films as a function of sampling time. (a) HDPE; (b) PET; (c) PVC.

## 4.4 Discussion

Physicochemical properties of three chemically distinct synthetic polymers were analysed over a 56-week period to assess their degradation in seawater. After exposing the surface morphology of all the tested plastics changed. Surface textures such as fractures, grooves, and large cracks appeared on HDPE and PET films, whereas PVC films displayed more well-defined pits. Similar surface morphologies changes have been reported on plastics sampled from beaches and seawater (Corcoran et al., 2009, Cooper and Corcoran, 2010, Zbyszewski and Corcoran, 2011, Fotopoulou and Karapanagioti, 2015, Cai et al., 2018, Da Costa et al., 2018, Tang et al., 2018). In all these accounts, the textures were attributed to chemical oxidation due to either solar or artificial UV irradiation. Though the plastics were not directly exposed to sunlight in this study as they were submerged in seawater, a small amount of UV radiations still penetrated (Figure 4.3.1). Additionally, the inorganic ions (e.g. sulphates, chlorides) present in the seawater can act as catalysts for plastic oxidation under reduced UV radiation which might have resulted in these morphological changes (Leonas, 1993, Da Costa et al., 2018). However, the low concentration of dissolved oxygen in the seawater reduces the photo-oxidation regardless of the amount of UV rays penetration (Biber et al., 2019). The gradual development of uneven surfaces for all the tested plastics also suggests peeling off the upper layers (Tang et al., 2018) and thus exposing the inner surfaces for further degradation. It is also plausible that the plastic surfaces might have undergone enzymatic hydrolysis due the presence of microorganisms (Chapter 3, Section 3.3.5) that can produce extracellular enzymes with the potential of degrading plastics (Balasubramanian et al., 2010, Vague et al., 2019). Such hydrolytic degradation reactions preferentially occur at the amorphous regions of the polymer (Donelli et al., 2009, Herrero Acero et al., 2011). Moreover, the inorganic ions including calcium or magnesium cations present in the seawater can also act as catalysts for these enzymatic hydrolysis reactions (Kawai et al., 2019).



Further insights into the surface properties of the plastics were determined by measuring their wettability (the ability of a liquid to spread out on a solid substrate). While immersed in seawater, plastics are known to change their surface properties due to water diffusion and restructuring of surface molecules in order to reduce the interfacial/surface energy of the plastics (Dussud et al., 2018). This phenomenon denotes to the hydrophobicity of the plastics. In contrast, the current study observed a significant change in the surface wettability of all tested plastics when exposed to the seawater. (Figure 4.3.5) The plastic surfaces became hydrophilic which was observed by the gradual decrease in the water contact angle (CA) of all the plastics over the exposure period. This is attributed to the attachment of microorganisms on the plastic surfaces (Chapter 3, Section 3.3.1) (Dussud et al., 2018) and subsequent surface degradation (Sudhakar et al., 2008, Yang et al., 2014, Yang et al., 2015, Nauendorf et al., 2016). A change in hydrophilicity further promotes biofilm attachment (Callow and Fletcher, 1994, Artham et al., 2009a, Dussud et al., 2018). Contrary to the water CA, there was a general increase in the formamide contact angle of the plastics, suggesting an increase in the interfacial energy (excess energy at the surface of plastics). This was in agreement with previous studies demonstrating that the colonisation of marine microorganisms changes the surface topography and increases the interfacial energy of PET films in seawater (Webb et al., 2009) and the interfacial rearrangements occur in the region between the surface and the colonising microorganisms (Busscher et al., 2010). Although seawater is more hydrophilic and due to alkaline in nature it can also induce surface modifications by alkaline hydrolysis therefore can increase the surface hydrophilicity of the polymers (Maekawa et al., 2006, Donelli et al., 2009). However, the development of biofilm on the plastic surfaces (Section 3.3.1) which contained potential biodegraders (section 3.3.5) because of their ability to produce hydrolytic enzymes (Danko et al., 2004, Ronkvist et al., 2009, Balasubramanian et al., 2010, Vague et al., 2019), it is more probable that the decrease in the contact angle and consequently increase in the degree of hydrophilicity of plastics was due to surface degradation by enzymatic hydrolysis (Sudhakar et al., 2008, Donelli et al., 2009)

The changes in tensile stress differed among the tested plastics. The tensile stress of HDPE decreased slowly but significantly over time, indicating that HDPE film demonstrated degradation in seawater. This is in agreement with previous seawater field-studies that reported a reduction in tensile strength of polyethylene films exposed over 40 weeks (O'Brine and Thompson, 2010) and 48 weeks (Heimowska et al., 2011). Mechanical damage of the PE macro chains may be also caused by the swelling and bursting of growing cells of the invading microorganisms or by macro-organisms in seawater (Heimowska et al., 2011).



Other studies showed that the changes in the tensile properties of PE were much faster when exposed in the air than seawater due to higher oxidation rate (Pegram and Andrady, 1989, Andrady, 1990, Andrady et al., 1993a, Biber et al., 2019). However, the slow change in the tensile properties of HDPE films in the current study is likely due to lowered temperatures and attenuated UV light in seawater which inhibits the oxidation. The apparent small changes in the tensile strength of all plastics also indicated that the bulk material had not changed to a large extent and could only be due the surface degradation as observed in SEM and contact angle analysis.

There was no significant change in the tensile stress of PET films, except for the late decrease between 42 and 56 weeks (Figure 4.3.6). This potentially suggests a comparative late onset of PET deterioration and shows that exposure time could be the limiting factor for its degradation within seawater. Previously, reduced degradation of PET has been observed in seawater. Heimowska et al. (2011) exposed PET for 48 weeks in the Baltic sea at the depth of 2 m and reported a non-significant change in its tensile properties. A long-term seawater exposure (600 days) study also reported no measurable loss of tensile properties for PET films (Biber et al., 2019). In addition to PET, the tensile stress tests performed to determine the changes in the mechanical properties of PVC films did not show any significant changes. It could be due to the deployment of the PVC samples two meters deep in the water column which reduced its susceptibility to photodegradation, even though it is very sensitive to UV radiation (Gewert et al., 2015, Bond et al., 2018). Moreover, the colonising microorganisms on the PVC films (Chapter 3, Section 3.3.1) may further reduce the UV irradiation by covering the surface and the shielding the plastic from UV light (O'Brine and Thompson, 2010, Welden and Cowie, 2017).

The investigation of chemical composition by ATR-FTIR indicated subtle changes. For PET exposed in seawater, a decrease in absorbance is observed for characteristics peaks at 1721 (C=O) and 1241  $\text{cm}^{-1}$  (C-O stretching) (Figure 4.3.8) relative to control. Such decline in band intensities has been previously observed and may indicate a change in the composition of the PET (Fotopoulou and Karapanagioti, 2015, Ioakeimidis et al., 2016). The decrease in the intensity of these peaks leads to the expectation of new peaks formation which, in fact, was observable. New peaks appeared at 970, 1120 and 1578  $\text{cm}^{-1}$  over the exposure time, suggesting the development of a new C-H, C-O and aromatic bond respectively (Coates, 2006). In contrast, Ioakeimidis et al. (2016) reported new peaks at 1435 and 620  $\text{cm}^{-1}$  corresponding to alkyne ( $\text{C}\equiv\text{H}$ ) and C-H bond, respectively were attributed to PET degradation in the marine environment. On the other hand, no new peaks were observed for

HDPE and PVC after seawater exposure in this study, suggesting that the degree of weathering was not sufficient to promote the formation of degradation products responsible for respective peaks. However, reduction in characteristic peaks was observed for both plastics along with peak broadening. This could be due to the chemical changes resulting from the bonding of specific elements (e.g. H, C) with other chemical groups of plastics and such changes have been demonstrated for plastic films after their exposure to microorganisms (Restrepo-Flórez et al., 2014, Skariyachan et al., 2017).

Despite the observable patterns within the FTIR spectra of plastics, no clear trends were detected for carbonyl index measurements. This could be attributed to the low oxidation within the seawater due to lower temperature and reduced UV radiation (Pegram and Andrady, 1989, Andrady, 1990, Andrady et al., 1993a, Biber et al., 2019). This limited oxidation could also be explained by the presence of *Cyanobacteria* on all the plastic surfaces (Chapter 3, Section 3.3.5) which could be involved in the oxidation of the polymers by increasing the rates of oxygen production and respiration (Bryant et al., 2016). However, the development of uneven surfaces (SEM observations) suggests that after a certain stage of degradation in seawater, the upper layer of the plastics becomes so brittle that it erodes and expose the new underlayer (Halle et al., 2017), which could explain why the carbonyl indices did not show any trends. Moreover, it could also be due the enzymatic hydrolysis of amorphous regions of polymers which could result in the change of polymer crystallinity (Donelli et al., 2009). The members of biofilm communities (e.g. *Pseudomonas*) reported in the previous chapter (Chapter 3, section 3.3.5) have the ability to produce extracellular enzymes which could degrade polymers by hydrolysis (Danko et al., 2004, Ronkvist et al., 2009, Balasubramanian et al., 2010, Vague et al., 2019). Within the plastic construct, the amorphous regions are more susceptible as they favour the hydrolytic attack of these enzymes (Donelli et al., 2009, Ronkvist et al., 2009).

## 4.5 Conclusions

This study analysed the long-term degradation of three widely used synthetic plastics in seawater (HDPE, PVC and PET). The results demonstrated that surface morphology and wetting properties of these plastics significantly changed over time. HDPE demonstrated the fastest rate of deterioration out of the three-plastic tested. However, the molecular composition and the bulk properties (e.g. tensile strength) of the samples did not change. It was attributed to nonlinear oxidation of plastics due to attenuated UV radiation in the water column, a lower average temperature and biofilm formation on the plastics. Therefore, the degradation plastics in the marine environment appeared to be more complex than predicted; as the carbonyl bond also did not change linearly with time. Due to the nonlinear changes in carbonyl bond, the indices presented here are of potential use for quantifying the exposure time of plastics only over a relatively limited time period.

# **Chapter 5: Biodegradation of Synthetic Plastics by Marine Consortia: A Respirometric Study**

## **5.1 Introduction**

In Chapters 3 & 4 it was demonstrated that the surface properties of plastic material changed significantly over time when submerged in seawater. This was attributed to the attachment of microorganisms (Chapter 3, Section 3.3.1) and the seawater chemistry. These studies also provided evidence that the colonising biofilm on the plastic surface could be playing an active role in degradation. However, these studies provided no direct proof of mineralisation due to microbial activity. Measuring the degradation of plastics in terrestrial environments is well established (Song et al., 2009, Briassoulis et al., 2010) with published international test methods and standards (e.g. BS EN 13432). In contrast, there is a lack of published test methods and standards to for evaluating the biodegradability of plastics within most marine environments (Tosin et al., 2012) and recreating the multitude of different parameters associated with a marine environment is challenging in laboratory environments (Harrison et al., 2018a). One approach to assess the plastic biodegradability is to simulate the marine (seawater) environment in a closed system (Pauli et al., 2017). Therefore, using laboratory simulation experiments, this chapters seeks to investigate the biodegradation of plastics in marine conditions.

Plastics are potential substrates for heterotrophic marine microorganisms including bacteria and fungi (Shah et al., 2008). The biodegradability of plastics depends on the physical state, crystallinity, molecular weight and the metabolic interactions within the plastic-attached biofilms (Gu, 2003, Artham et al., 2009b, Harrison et al., 2011). Generally, the biodegradation of plastics within marine environments comprises of four steps including: biodeterioration; biofragmentation; assimilation and mineralisation. The first step, biodeterioration, involves biofilm formation on the plastic surface which modifies the physical, mechanical and chemical properties of the plastics. The second step is the biofragmentation in which the plastics are cleaved into smaller units (e.g. oligomers and monomers) by the extracellular enzymes of colonising microorganisms. The next step is assimilation where the oligomers are transported inside the microbial cells to produce

energy, new biomass and various metabolites. The last step of the biodegradation process is mineralisation which refers to excretion of simple (e.g. CH<sub>4</sub>) and completely oxidized metabolites (e.g. CO<sub>2</sub>, H<sub>2</sub>O) to the extracellular surroundings (Lucas et al., 2008, Dussud and Ghiglione, 2014, François-Heude et al., 2014).

The ability of microorganisms to degrade plastics have been known for several decades, with the first study in 1968 identifying bacterial isolates as potential plastic degraders (Booth et al., 1968). However, the biodegradation of plastics within the marine environments only became a focus of research a couple of decades later (Palmisano and Pettigrew, 1992). So far, only a few studies have analysed the biodegradation potential of a wide range of plastics in the marine environments (Table 1.4.1, Chapter 1: Section 1.4.3). These studies mainly focused on the isolation and *in vitro* biodegradability testing of microbial strains from the marine environments. The isolated microorganisms showed the biodegradation potential of the plastics (Urbanek et al., 2018). For example, Balasubramanian et al. (2010) isolated bacterial strains belonging to genera *Pseudomonas* and *Arthrobacter* from Gulf of Mannar, with HDPE degradation ability. Similarly, bacterial genera of *Kocuria*, *Bacillus* and *Rhodococcus* had also been reported for the biodegradation of polyethylene (Harshvardhan and Jha, 2013, Auta et al., 2017, Auta et al., 2018). Moreover, marine fungi e.g. *Aspergillus* and *Zalerion* sp. have also been identified as plastic degraders (Devi et al., 2015, Paço et al., 2017). Although these studies claim to assess the microbial degradation of plastics in marine environments, they provide no direct experimental data on microbial assimilation of plastic. The potential biodegradation was assessed either by weight loss measurements or by determining the changes in morphological, mechanical and chemical properties of plastics, which do not necessarily prove the biodegradation process (Shah et al., 2008). Therefore, little is known about the complete assimilation and mineralisation of plastics by marine microbes.

Whilst the marine microbes have demonstrated the capabilities to degrade plastics, the rate of degradation was low, even in optimised laboratory conditions (Krueger et al., 2015, Debroas et al., 2017). It was attributed to the large size of plastic molecules, higher chemical stability and lower bioavailability of plastic materials to microbial cells (Alexander, 1975, Andrady, 1994). Additionally, most of the studies used single microbial strains (Sekiguchi et al., 2009, Balasubramanian et al., 2010, Harshvardhan and Jha, 2013, Auta et al., 2017, Auta et al., 2018, Giacomucci et al., 2019) that can also limit the plastic biodegradation process as the mineralisation of plastics is a complicated process and usually conducted by consortium of species with functional complementarity (Syranidou et al., 2017).

Therefore, the specific aims of this Chapter are:

1. To isolate three marine bacterial consortia depending on their ability to biodegrade three chemically distinct synthetic plastics;
2. To assess the capability of the consortia to degrade plastics in a simulated marine environment using a respirometric system;
3. To measure changes in the physicochemical properties of plastic after the biodegradation.

## **5.2 Materials and methods**

### **5.2.1 Seawater Sampling**

In order to isolate the microorganisms from the marine environment, natural seawater samples were collected from Skegness beach, UK (53.137844 °N; 0.351808 °E), on the 20<sup>th</sup> of April 2017. These samples were previously used in Chapter 2 to study the effects of plastics on marine microbial communities in seawater. Full details of the sample collection, handling and storage for subsequent analysis are included in the methods section of Chapter 2 (2.2.1).

### **5.2.2 Enrichment of Marine Consortia**

A three-step enrichment method was used to isolate three unique marine bacterial consortia with the potential to degrade three chemically distinct synthetic plastics; high-density polyethylene (HDPE), polyethylene terephthalate (PET) and polyvinyl chloride (PVC). At first, the enrichment was carried out in natural seawater. For this, 100 mL of natural seawater was transferred aseptically to three 250 mL capacity autoclaved Erlenmeyer flasks individually, one for each plastic-type. To these flasks, one gram (g) of plastics (each type to separate flask) was added. Prior to the use, the plastics were ground using a CryoMill (Retsch GmbH, Haan, Germany) and sterilised with 70 % ethanol and subsequently air-dried in laminar flow hood. Following the addition of seawater and the plastics, the flasks were sealed with cotton wool to prevent contamination and then incubated on a rotary shaker (120 rpm) at room temperature for 30 days. In the second step, the enrichment was performed in marine broth. For this, 5 ml of previously enriched natural seawater was used as inoculum and transferred to three new sterile flasks separately. Each flask contained 100 mL of marine broth and one gram of single plastic-type. The marine broth was prepared with deionised water and contained the following (per 1000 mL): 19.45 g of NaCl, 5.9 g of MgCl<sub>2</sub>; 5 g of Peptone; 3.24 g of NaSO<sub>3</sub>; 1.8 g of CaCl<sub>2</sub>; 1 g of yeast extract; 0.55 g of KCl; 0.16 g of

NaHCO<sub>3</sub>; 0.10 g of ferric citrate; 0.08 g of KBr; 0.034 g of SrCl<sub>2</sub>; 0.022 g of H<sub>3</sub>BO<sub>3</sub>; 0.008 g of K<sub>2</sub>HPO<sub>4</sub>; 0.004 g of sodium silicate and 0.001 g of NH<sub>4</sub>NO<sub>3</sub>. The pH of the broth was adjusted to 7.0 using 1M HCl and then sterilised by autoclaving at 122 °C for 20 min. The inoculation was performed in a laminar flow hood to minimise the risk of contamination. The flasks were then sealed and incubated on a rotary shaker (120 rpm) at room temperature for another 30 days. Finally, the same procedure used for the second step was repeated for the third step of the enrichment method and the marine broth from the second enrichment was used as inoculum for this step.

### **5.2.3 Selection and Isolation of Marine Bacteria with the Plastic Degrading Ability**

In order to develop a unique consortium for each plastic-type, bacterial isolates with plastic degrading ability were first selected. For this, the cultures obtained after the enrichment process was spread across plates containing carbon-free minimal salt agar medium. The agar plates contained the following (per 1000 mL of deionised water): 15 g of agar; 1 g of NaCl; 1 g of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>; 1 g of K<sub>2</sub>HPO<sub>4</sub>; 0.2 g of KH<sub>2</sub>PO<sub>4</sub>; 0.025 g of yeast extract; 0.01 g of ZnSO<sub>4</sub>·7H<sub>2</sub>O; 0.01 g of FeSO<sub>4</sub>·7H<sub>2</sub>O; 0.005 g of H<sub>3</sub>BO<sub>3</sub>; 0.002 g of CaCl<sub>2</sub>·2H<sub>2</sub>O; 0.001 g of CuSO<sub>4</sub> and 0.001 g of MnSO<sub>4</sub>·H<sub>2</sub>O. The enriched cultures (0.5 mL) of each plastic-type was spread homogeneously across the surface of the agar plates, which were subsequently covered with plastic film (20 mm × 20 mm). The plates were then sealed with parafilm and incubated at 30 °C for 30 days. The growth on the plastic films was then selected for subsequent isolation and purification of bacterial isolates.

To obtain pure isolates, the plastic films with growth were transferred into separate sterile flasks containing 100 mL of fresh marine broth. The flasks were incubated at 30 °C for 7 days and the successfully growing cultures (0.1 mL) were pipetted on marine agar plates. After incubation of 7 days, the colonies formed on the marine agar plates were shifted to new plates with fresh agar medium to obtain pure colonies of the isolates. For this, a streak plate method was used, which is a quadrant streak technique that facilitates sequential dilution of the original inoculum over the entire surface of the agar plate (Sanders, 2012). The individual pure colonies were then selected for molecular identification of isolates.

### **5.2.4 Molecular Identification of Isolates**

In order to identify the isolates, sequencing of the 16S rRNA gene was performed (hypervariable regions V1-V9). For this, the bacterial universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTAGGACTT-

3') (Lane, 1991) were used. DNA was not extracted prior to PCR, instead, colony PCR was performed to amplify the 16S rRNA gene of cultured isolates. For this, amplification was carried out using a single colony in a 50 µL reaction mixture containing 250 nM of each primer, 5 µL of 5× Taq buffer, 2 mM MgCl<sub>2</sub>, 0.1 mM of each dNTP and 1.75 U of Taq (New England Biolabs, MA). PCR conditions were as follows: initial denaturation for 10 mins at 94 °C to promote cell lysis; followed by 35 cycles at 94 °C for 1 min; annealing at 55 °C for 40 sec; extension at 72 °C for 1 min and a final extension at 72 °C for 10 min. All PCR products were verified on a 2 % (w/v) agarose gel (Sigma, Aldrich, Germany) by electrophoresis with a DNA marker, 2-Log DNA Ladder (New England Biolabs, MA). The PCR products were then purified using co-precipitant pink (Bioline, London, UK) protocol according to the manufacturer's instructions.

The purified PCR products were sequenced at Eurofins genomics, Germany using Sanger sequencing, which was performed using a 3730xl DNA Analyser. Sequencing was undertaken in both forward and reverse direction using respective primers. Sequencing data in the form chromatograms were analysed using the software BioEdit v7.2.5 (Hall, 1999, Hall et al., 2011) to identify the high-quality DNA regions. Moreover, a contig (overlapping DNA fragments that in combination represent a consensus region of DNA) was prepared for each isolate using sequences of the 27F and 1492R amplifications. The contigs were aligned with organisms present on a DNA sequences database (GenBank) using the Basic Local Alignment Search Tool (BLAST) (Johnson et al., 2008), developed by National Center for Biotechnology Information (NCBI), USA. This analysis was performed to identify the most closely related species to each isolate and their % homology with these species.

#### **5.2.5 Biodegradation Test**

To quantify the biodegradation of synthetic plastics by the isolated bacterial consortia, a respirometric experiment was carried out in an optimised aerobic marine environment. For this, a bespoke respirometric system was developed and the test was performed according to the guidelines of ASTM D 6691-09 (ASTM-International, 2009). This test method is used to determine the degree of aerobic biodegradation of plastic materials exposed to isolated marine consortia. The test mixture contains an inorganic medium (artificial seawater), the organic test material (plastics) pre-selected marine microorganisms as the inoculum. The mixture is aerated with carbon-dioxide-free air over a period of time depending on the biodegradation kinetics, but not exceeding 6 months according to the standard guidelines.



### 5.2.5.1 Respirometric System

A respirometric system or respirometer is a device used to test and/or quantify the respiration activity of living organisms by either focusing on oxygen (O<sub>2</sub>) consumption, CO<sub>2</sub> evolution or both (Kijchavengkul et al., 2006). Generally, there are three major components of a respirometer: (1) an air supply, (2) an air-tight closed vessel containing the living test organisms, and (3) a measuring device, to measure the quantity of O<sub>2</sub> consumption and/or CO<sub>2</sub> evolution (Kijchavengkul et al., 2006). The respirometric system can also be adapted and used to quantify the amount of CO<sub>2</sub> gas evolved from the aerobic biodegradation of plastic materials (ASTM-International, 2009).

Based on ASTM D 6691-09 (ASTM-International, 2009), a closed system respirometer was used to measure CO<sub>2</sub> evolved from respirometer vessels using an infrared CO<sub>2</sub> analyzer. This respirometric system consisted of seven major components: (1) an air pump; (2) flow multiplexers; (3) respirometric vessels; (4) manifold valves; (5) CO<sub>2</sub> analyzer; (6) control software and (7) water bath. Ambient air was pushed through a pump (SS4 Sub-Sampler, Sable Systems International, NV, USA) to flow multiplexers (RM-8, Sable Systems International), which allowed selection of one channel at a time for further pushing of the air to a single respirometric vessel, thus allowed the build-up of CO<sub>2</sub> in other vessels. At the same time, the CO<sub>2</sub> in the headspace moved to manifold valves to prevent developing of hypercarbia condition between the readings. The manifold valves switched according to flow multiplexers and directed the exhaust CO<sub>2</sub> from vessels to the infrared gas analyser (CA-10A, Sable Systems International). Prior to and during the experiment, the gas analyser was calibrated frequently using a zero CO<sub>2</sub> gas (pure nitrogen) and a known amount of pure CO<sub>2</sub> (0.2 % CO<sub>2</sub>). All these components were controlled by ExpeData software (v 1.8.5) which also recorded the concentration of CO<sub>2</sub> of each vessel every two hours. A water bath was used to control the temperature of the respirometric vessels at 30 °C (Figure 5.2.1 a).

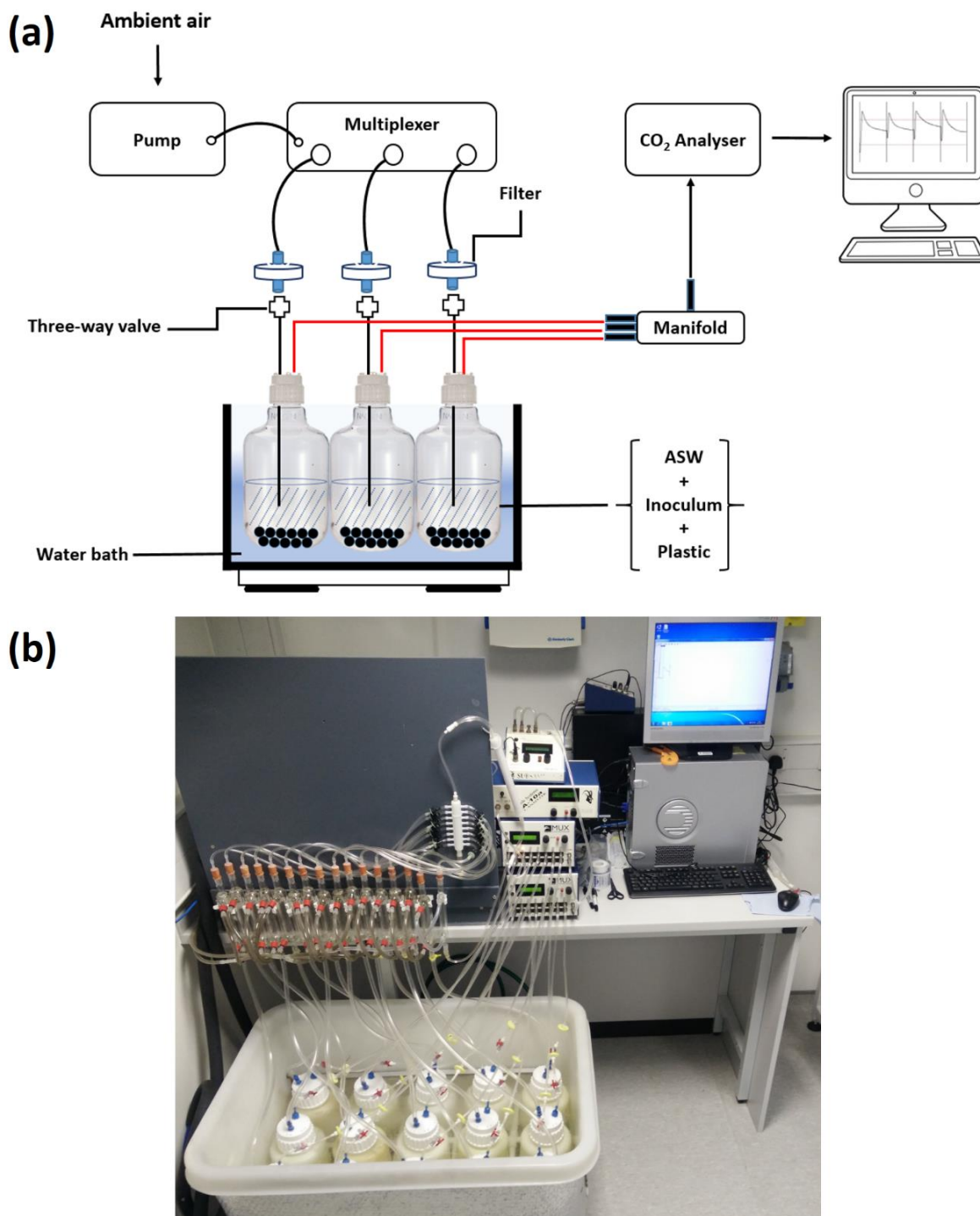


Figure 5.2.1 Experimental set-up for biodegradation test. (a) Schematic diagram of respirometric system components; (b) The original laboratory set-up of the respirometric system

#### 5.2.5.2 Preparation of Inoculum

The focus of this work was to develop a unique marine consortium for each plastic-type and assess its biodegradation potential. Therefore, the inoculum was prepared separately for each plastic-type by using previously isolated bacteria that were selected due to their growth on respective plastic after enrichment. For this, each isolate was grown separately in 50 mL of autoclaved marine broth for 7 days at 30 °C. The cultures (10 mL) of each isolate (according

to plastic-type) were then placed into a single sterile flask with cap and mixed together by using vortex. For reference material and blank (with no testing material), the inoculum was prepared by mixing all the isolates irrespective to enrichment source.

### 5.2.5.3 Preparation and Inoculation of Respirometry Vessels

For the respirometric experiment, 4 L capacity Nalgene (Fisher Scientific, Loughborough, UK) bottles were used as respirometric vessels. The vessels were first sterilised along with lids and the attached tubes prior to setting up the experiment. These vessels were not autoclavable, therefore, a two-step wet sterilisation was performed. In the first step, the vessels and the accessories were immersed in 1% virkon (a strong disinfectant) for 10 min then washed with distilled water and dried. In the second step, the vessels were thoroughly rinsed with 70% ethanol and closed the lids. The vessels were then filled with 2000 mL of a test medium, artificial seawater (ASW). It was prepared with deionised water and contained the following (per 1000 mL): 24.53 g of NaCl; 5.20 MgCl<sub>2</sub>; 4.09 g of Na<sub>2</sub>SO<sub>4</sub>; 1.16 g of CaCl<sub>2</sub>; 0.695 g KCl; 0.201 g of NaHCO<sub>3</sub> 0.101 g of KBr; 0.027 g of H<sub>3</sub>BO<sub>3</sub>; 0.025 g of SrCl<sub>2</sub> 0.003g of NaF; 99.4 µg of Ba(NO<sub>3</sub>)<sub>2</sub>; 34 µg of Mn(NO<sub>2</sub>)<sub>2</sub>; 30.8 µg of Cu(NO<sub>3</sub>)<sub>2</sub>; 9.6 µg of Zn(NO<sub>3</sub>)<sub>2</sub>; 6.6 µg of Pb(NO<sub>3</sub>)<sub>2</sub> and 0.49 µg of AgNO<sub>3</sub>. The pH was adjusted to 8.2 with 0.1N NaOH solution and then autoclaved at 122 °C for 20 min. Additionally, 5 g of the test (HDPE, PET and PVC; in both powder and film form) and reference (cellulose; powder only) specimens were added to vessels. The samples were sterilised using 70 % ethanol and subsequently air-dried in laminar flow hood prior to placement into vessels. Three replicates were set up for each plastic, reference and blank (with no additional material). Finally, the vessels were inoculated with the 5 mL of previously prepared inocula and attached the vessels to respirometer after placing them in a water bath set to 30 ± 2 °C (Figure 5.2.1 b). The biodegradation test was performed for 6 months as per ASTM D 6691-09 (ASTM-International, 2009) guidance.

### 5.2.5.4 Calculations

To calculate the level of ultimate biodegradation, the theoretical amount of carbon dioxide was first determined using the Equation 5.2.5.1

$$ThCO_2 = X_c \times \frac{MCO_2}{AMC} \times m \quad \text{Equation 5.2.5.1}$$

where  $X_c$  is the organic carbon content of the test materials that were determined by using the method described in Chapter 2 (Section 2.2.3.2),  $MCO_2$  is the molecular mass of carbon dioxide (44.0095 g/mol) and  $AMC$  is the atomic mass of carbon (12.0107 g/mol). Furthermore,  $m$  is the total dry solids, in grams, in the test material introduced into the vessels at the start of the test and was determined using Equation 5.2.5.2

$$m = WW \times \left(\frac{DW}{100}\right) \quad \text{Equation 5.2.5.2}$$

where  $WW$  is the wet weight and  $DW$  is the dry weight. It is the weight of test material that remains after drying the sample overnight at 105 °C. The measured amounts of  $X_c$ ,  $WW$  and  $DW$  are presented in Table 5.2.1.

Table 5.2.1 The total organic carbon and the wet and dry mass of the test materials used for the study.

Polymers	Total organic carbon (gram C per gram)	Wet weight (g)	Dry weight (%)
Cellulose	0.43 ± 0.003	5	94.22 ± 0.79
HDPE	0.84 ± 0.001	5	98.99 ± 0.35
PET	0.61 ± 0.012	5	98.86 ± 0.45
PVC	0.37 ± 0.015	5	99.36 ± 0.35

Finally, percent carbon loss or rate of biodegradation ( $D_t$ ) was calculated using the Equation 5.2.5.3

$$D_t = \frac{\sum(CO_2)_{test} - \sum(CO_2)_{blank}}{ThCO_2} \times 100 \quad \text{Equation 5.2.5.3}$$

where  $\sum(CO_2)_{test}$  is the cumulative amount of carbon dioxide evolved (in grams) in each respirometric vessel containing test material,  $\sum(CO_2)_{blank}$  is the cumulative amount of carbon dioxide evolved (in grams) in blank vessels and  $ThCO_2$  is the theoretical amount of carbon dioxide evolved by the test material.

## 5.2.6 Analysis of Physicochemical Properties

After the biodegradation test, the morphological analysis of plastic films was performed using a Field Emission Gun- Scanning electron Microscope (FEG-SEM) (ZEISS Supra; 55-VP; Zeiss Micro-imaging, Gottingen, Germany), which was operated with an accelerating voltage of 3 kV and a 7 - 10 mm working distance. The samples were prepared by fixing in

2.5 % glutaraldehyde and 1 % osmium tetroxide (OsO<sub>4</sub>) solution. Subsequent dehydration of samples was carried out in graded (30, 50, 70, 90 and 100 %) ethanol. A full detailed description of the method used to prepare and visualise samples for SEM can be found in Chapter 3 (Section 3.2.3).

In order to examine the changes in the molecular structure, FTIR analysis was carried out using a Nexus, Thermo Nicolet (USA) FTIR spectrophotometer coupled with Specac Golden Gate ATR device. The plastic films were analysed within the range of 400-4000 cm<sup>-1</sup> wavenumber using a spectral resolution of 2 cm<sup>-1</sup>. The analysis was performed on plastic films before (control) and after (with biofilm and cleaned) the biodegradation test. For cleaning the plastic attached biofilms, previously described (in Chapter 4, Section 4.2.2 washing method with 2 % (w/v)) SDS solution was employed. Moreover, the carbonyl index was calculated as a proxy for degradation and change in the molecular composition of plastics (see Chapter 4, Section 4.2.3.3.1 for full details on carbonyl index measurements).

## **5.3 Results**

### **5.3.1 Preliminary Screening of Plastic Degrading Marine Bacterial Isolates**

Screenings for plastic degrading bacterial isolates were performed in terms of their colonisation on the HDPE, PET and PVC film in the carbon-free minimal salt media agar plates. A visible microbial growth was observed after spreading the enriched cultures on agar plates which were then covered with respective plastic films (Figure 5.3.1). The examination of growth on HDPE film revealed translucent colonies on the surface, with growth spreading out from a high concentration around the film in the middle (Figure 5.3.1 a). Similarly, a wide-spread growth was observed around the PET film but in contrast to HDPE, the growth was relatively thick and opaque colonies were visible on the PET surface (Figure 5.3.1 b). For PVC film, the enriched culture grew as thin, translucent colonies on the surface (Figure 5.3.1 c). All of these growths on plastic films were individually used to isolate bacteria using the method outlined in Section 5.2.3, which subsequently formed a unique consortium for each plastic-type.

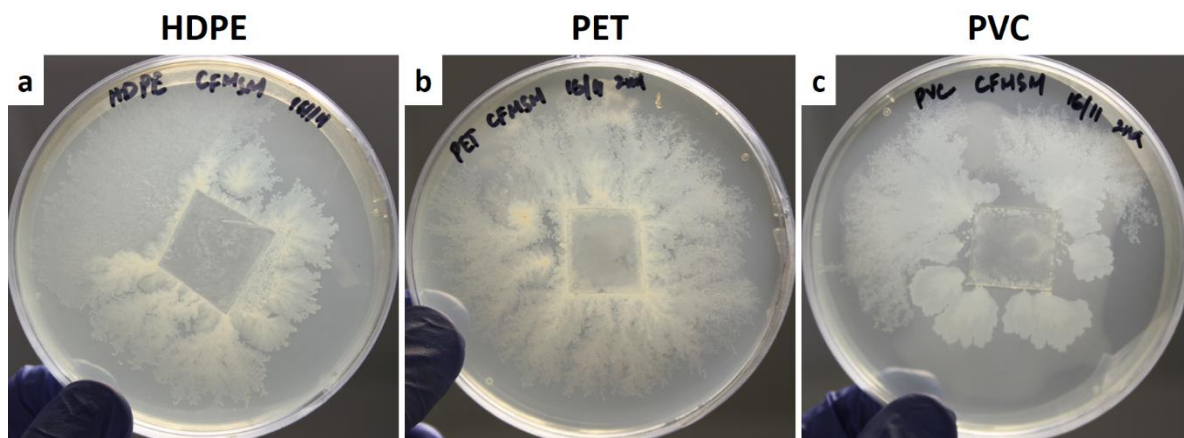


Figure 5.3.1 Mixed bacterial growth on the surface of plastics. (a) HDPE, (b) PET and (c) PVC

### 5.3.2 Identification of Isolates

A total of 19 bacterial cultures were isolated by selecting colonies that formed after inoculating the marine broth with biofilms from the different plastics. Five distinct bacterial colonies were isolated from HDPE, six from PET and eight from PVC. The isolates were taxonomically identified to the genus level using the NCBI Blast database (Table 5.3.1). Each of the isolates was closely related (99 % similar) to species that have been previously isolated either from seawater, marine sediments, wastewater or soil environments (Table 5.3.1). Additionally, one of the isolates from the PET film belonged to the *Bacillus* genus which is closely related to *Bacillus massiliosenegalensis* that is found in the human gut (Lagier et al., 2012).

There was a commonality between the bacteria isolated from the different plastics, for example, bacteria belonging to the genera *Salinibacterium* and *Bacillus* were isolated from each plastic-type. In addition, bacteria closely related to *Rhodococcus qingshengii* were isolated from HDPE and PVC; *Vibrio* was found on both PET and PVC films. However, distinct bacteria were also isolated for each-plastic type. This included isolates closely related to *Pseudoalteromonas hotoensis* (for HDPE); *Bacillus massiliosenegalensis*; *Pseudoalteromonas prydzensis* (for PET); *Epibacterium scottomollicae*; *Cellulophaga geojensis*; *Pseudomonas guineae* and *Microbacterium maritopicum* (for PVC) (Table 5.3.1).

Table 5.3.1 The taxonomical identification of marine bacteria isolated from the surface of plastics after the enrichment procedure.

Plastic	Isolate	Closest Blast Match (NCBI nt database)	Source of the closest relative
HDPE	AN-HDPE-01	<i>Pseudoalteromonas hotoensis</i>	Coastal seawater (Chi et al., 2014)
HDPE	AN-HDPE-02	<i>Oceanisphaera sediminis</i>	Marine sediment (Shin et al., 2012)
HDPE	AN-HDPE-03	<i>Rhodococcus qingshengii</i>	Wastewater treatment facility (Wang et al., 2010)
HDPE	AN-HDPE-04	<i>Salinibacterium amurskyense</i>	Seawater (Han et al., 2003)
HDPE	AN-HDPE-05	<i>Bacillus licheniformis</i>	Soil (Veith et al., 2004)
PET	AN-PET-11	<i>Salinibacterium amurskyense</i>	Seawater (Han et al., 2003)
PET	AN-PET-12	<i>Oceanisphaera donghaensis</i>	Marine sediment (Park et al., 2006b)
PET	AN-PET-13	<i>Bacillus massiliosenegalensis</i>	Human gut (Lagier et al., 2012)
PET	AN-PET-14	<i>Bacillus licheniformis</i>	Soil (Veith et al., 2004)
PET	AN-PET-15	<i>Vibrio pacinii</i>	Various marine organisms (Thompson et al., 2001)
PET	AN-PET-16	<i>Pseudoalteromonas prydzensis</i>	Antarctic coastal areas (Bowman et al., 1997)
PVC	AN-PVC-21	<i>Bacillus licheniformis</i>	Soil (Veith et al., 2004)
PVC	AN-PVC-22	<i>Rhodococcus qingshengii</i>	Wastewater treatment facility (Wang et al., 2010)
PVC	AN-PVC-23	<i>Salinibacterium amurskyense</i>	Seawater (Han et al., 2003)
PVC	AN-PVC-24	<i>Vibrio pacinii</i>	Various marine organisms (Thompson et al., 2001)
PVC	AN-PVC-25	<i>Epibacterium scottmollicae</i>	Marine electroactive biofilm (Vandecastelaere et al., 2008)
PVC	AN-PVC-26	<i>Cellulophaga geojensis</i>	Marine sand (Park et al., 2012)
PVC	AN-PVC-27	<i>Pseudomonas guineae</i>	Antarctic environment (Bozal et al., 2007)
PVC	AN-PVC-28	<i>Microbacterium maritropicum</i>	Swamp forest (Park et al., 2006a)

### 5.3.3 Respirometric Analysis

Biodegradation tests were initially set to perform for 6 months performed according to ASTM D 6691-09 (ASTM-International, 2009). But the carbon dioxide evolution did not reach the plateau stage after 6 months therefore, the incubation period was extended to 7 months. At the end of the test, the mean amount of cumulative carbon dioxide of each plastic-type, reference material (cellulose) and the blank was calculated and is presented in Figure 5.3.2. For this, the data collected in ppm measurements were first converted to the volume percentage of CO<sub>2</sub>. It was then converted to grams based on the flow rate of ambient air to the system. After 210 days of incubation, HDPE samples displayed the highest mean cumulative CO<sub>2</sub> of 2.45 ± 0.07 g and the mean cumulative CO<sub>2</sub> evolved for PVC, PET,

cellulose and blank vessels were  $2.21 \pm 0.13$ ,  $1.84 \pm 0.51$ ,  $1.41 \pm 0.11$  and  $1.13 \pm 0.15$  g, respectively (Figure 5.3.2).

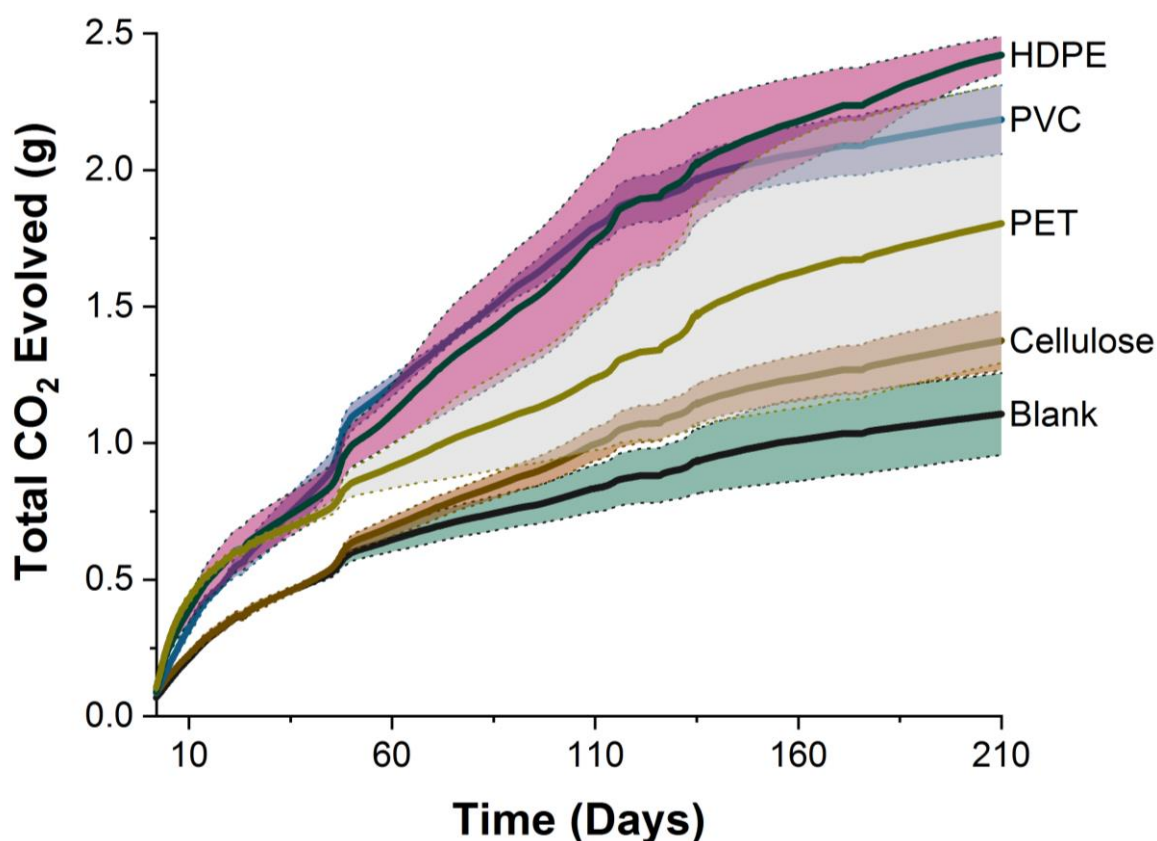


Figure 5.3.2 Total carbon dioxide (CO<sub>2</sub>) evolved from Blank, Cellulose, HDPE, PET and PVC treatment over a seven-month incubation in artificial seawater along with respective marine bacterial consortia. The shaded areas represent  $\pm 1$  standard deviation of the mean.

Next, the percentage carbon loss (% biodegradation) of each test material was calculated using Equation 5.2.5.3, which is the relationship between the actual amount of CO<sub>2</sub> evolved from the test material and the theoretical amount of CO<sub>2</sub> (maximum CO<sub>2</sub> that can be evolved from the test material). Following incubation with respective consortia, the biodegradation of each synthetic plastic appeared to start without an apparent lag-phase (Figure 5.3.3). After 210 days of the test, the PVC showed highest biodegradation percentage (16.26 %) whereas the per cent carbon loss for HDPE and PET was 8.71 and 6.51 %, respectively. In addition, the cellulose vessels showed mean biodegradation of only 3.74 % (Figure 5.3.3).



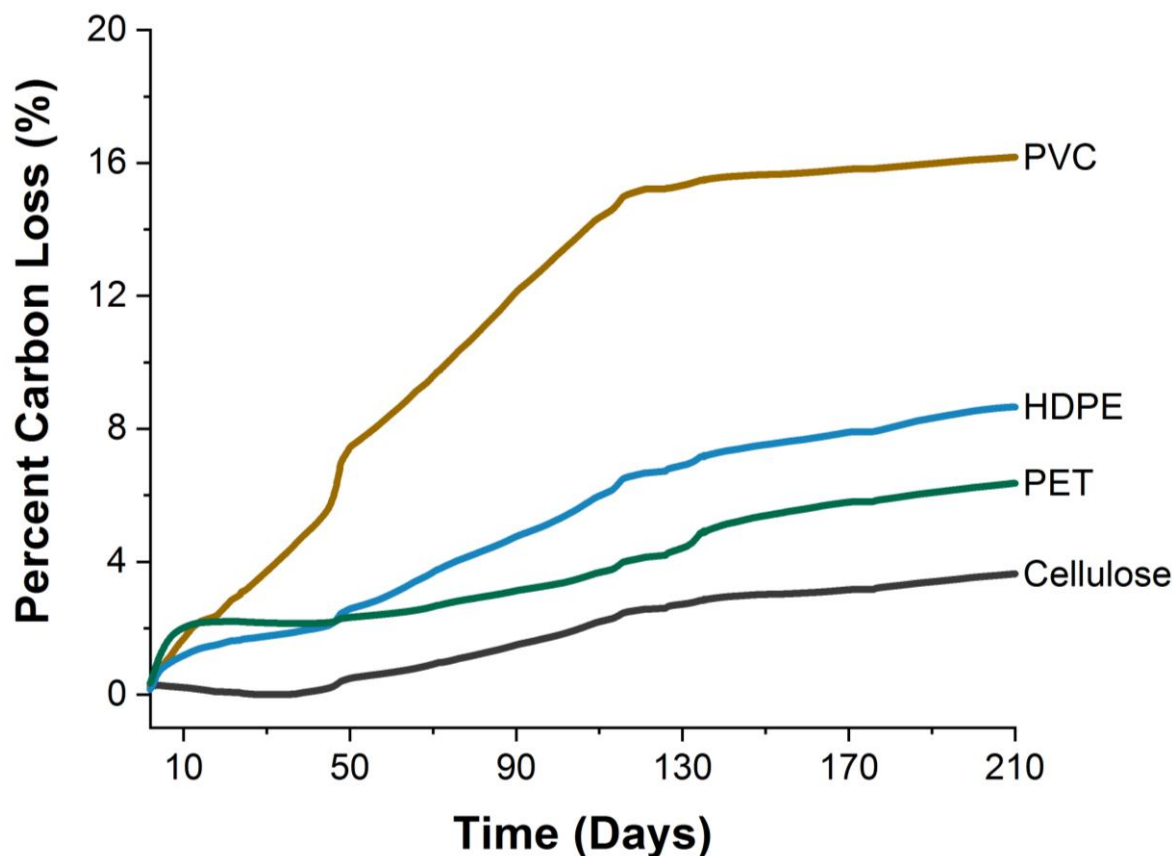


Figure 5.3.3 Percentage of estimated carbon dioxide that was evolved from Cellulose, HDPE, PET and PVC treatment over a seven-month incubation in artificial seawater along with respective marine bacterial consortia.

#### 5.3.4 Surface Morphology of the Plastics

Scanning electron microscopy was used to examine attached biofilm and the physical surface of the three plastic-types (HDPE, PET and PVC) before and after the respirometric test. SEM analysis showed morphologically diverse biofilm and changes in the surface topography of all plastic types (Figure 5.3.4). The comparison of control and microbially treated HDPE films in the respirometer revealed differences on the polymer surface. The control HDPE film had a smooth and featureless surface (Figure 5.3.4 a), however, after incubation with unique HDPE marine consortium, many fissures were observed throughout the surface and these microcracks were more evident around the attached bacterial cells (Figure 5.3.4 b).

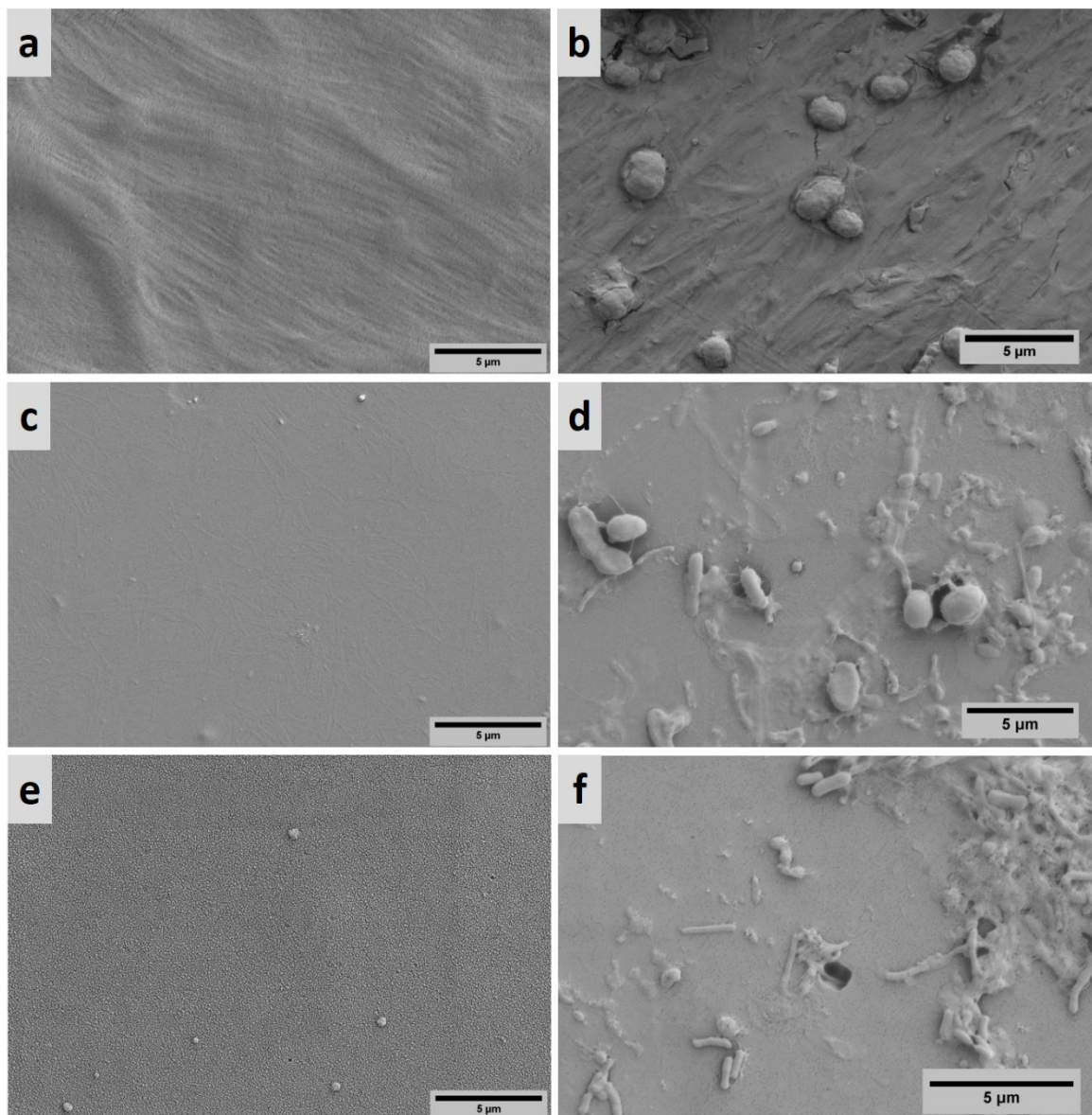


Figure 5.3.4 Scanning electron micrographs showing the attached biofilm and topography of plastic films. (a) HDPE control, (b) HDPE after (t-210 days), (c) PET control, (d) PET after (t-210 days), (e) PVC control, (f) PVC after (t-210 days) of the respirometric test. Scale bar = 5  $\mu$ m.

The SEM examination of the control PET film revealed a uniform smooth surface (Figure 5.3.4 c). Following the biodegradation test, the surface of PET films was not changed substantially and presented uniformity with some localized deterioration in the form of small pits. It was noted that bacterial cells attached on to these pits using extracellular organelles (e.g. pili), indicating their adhering capabilities and potential utilisation of PET (Figure 5.3.4 d).

For PVC, the control films presented a relatively uniform surface with a few spheroid shape granules (Figure 5.3.4 e). However, the PVC films collected after the biodegradation test appeared to have a degraded surface containing large pits (Figure 5.3.4 d). The formation of pits was indicative of polymer fragmentation during the test. In addition, a dense

heterogeneously distributed biofilm was also observed on the PVC film with some of the biofilm cells found growing inside the pits (Figure 5.3.4 f).

### 5.3.5 Changes in ATR-FTIR Spectra

The attenuated reflectance (ATR) Fourier transform infrared (FTIR) spectroscopy was used to analyse the chemical changes after the respirometric biodegradation test. The ATR-FTIR spectra of all three plastic types (HDPE, PET, and PVC) before and after the respirometric test were plotted in a comparative way in Figure 5.3.5, Figure 5.3.6 and Figure 5.3.7 respectively.

For control HDPE film, characteristic peaks were identified at wavenumbers of 717, 730, 1462, 1472, 2846 and 2915  $\text{cm}^{-1}$  corresponding to vibrating  $\text{CH}_2$  groups, bending  $\text{CH}_2$  groups and C–H stretching (Figure 5.3.5). These native peaks were discussed in detail in Chapter 4 (Section 4.3.5). After the biodegradation test, the ATR-FTIR analysis of HDPE with biofilm showed the formation of new peaks at the wavenumbers of 1120 and 1186  $\text{cm}^{-1}$ , suggesting the presence of alkoxy and acyl C–O stretching bands. In addition, a wide peak, centred at 3390  $\text{cm}^{-1}$  was also observed, indicating the presence of OH functional group. Similarly, the cleaned HDPE film also showed peaks at 1120 and 1186  $\text{cm}^{-1}$  but the peak at 3390  $\text{cm}^{-1}$  was disappeared after washing (Figure 5.3.5).

The ATR-FTIR spectra of PET had six characteristic peaks at the wavenumbers of 722, 871, 1094, 1241, 1410 and 1712  $\text{cm}^{-1}$  which were attributed to C–H bending, C–O stretching, C=C stretching and C=O stretching respectively. More details on these peaks were discussed in Chapter 4 (Section 4.3.5). The ATR-FTIR spectra of PET samples collected after a 210-day respirometric study was different from that of control (Figure 5.3.6). For PET with biofilm, the corresponding spectra showed the formation of peaks at 2359 and 2970  $\text{cm}^{-1}$ . These peaks were attributed to uneven surface, background  $\text{CO}_2$  and C–H (asymmetrical and symmetrical) stretching. Moreover, a negligible broadened peak was also observed in the region of 3300–3400  $\text{cm}^{-1}$ . A close look at the data revealed that the peak intensity was highest at 3390  $\text{cm}^{-1}$ , corresponding to OH functional group. In contrast, the spectra of cleaned PET presented no peak at 2359 and 3390  $\text{cm}^{-1}$  but it retained the peak at 2970  $\text{cm}^{-1}$ . Moreover, a new peak was also observed for cleaned PET at 1576  $\text{cm}^{-1}$ , corresponding to C=C stretching (Figure 5.3.6).

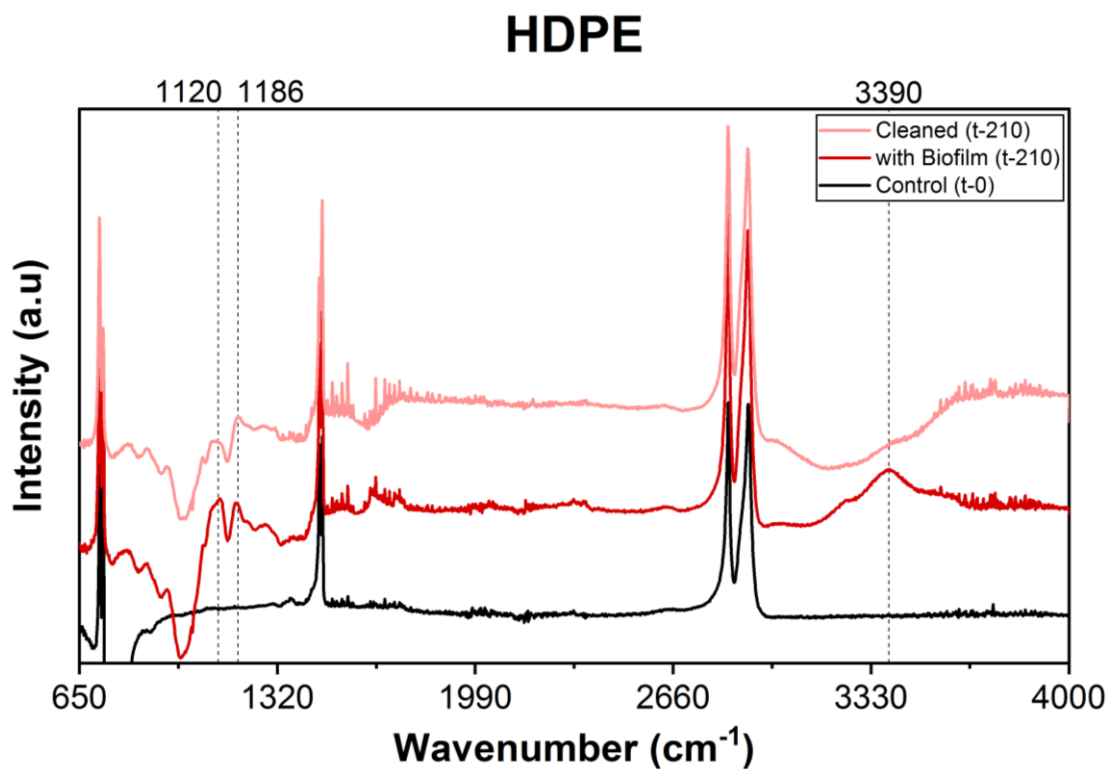


Figure 5.3.5 ATR-FTIR spectra of HDPE films. Comparative spectra of HDPE before (t-0) and after (t-210) days (with biofilm and cleaned) of the respirometric test. The intensity of peaks recorded as arbitrary units (a.u.).

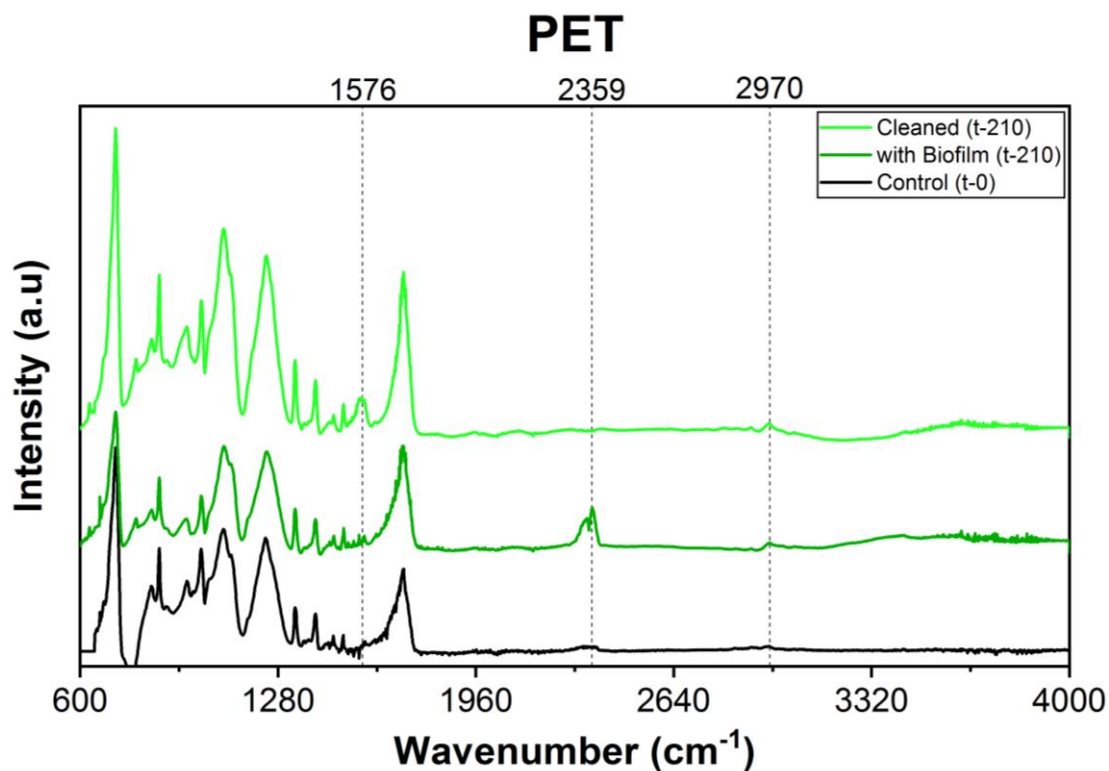


Figure 5.3.6 ATR-FTIR spectra of PET films. Comparative spectra of PET before (t-0) and after (t-210) days (with biofilm and cleaned) of the respirometric test. The intensity of peaks recorded as arbitrary units (a.u.).

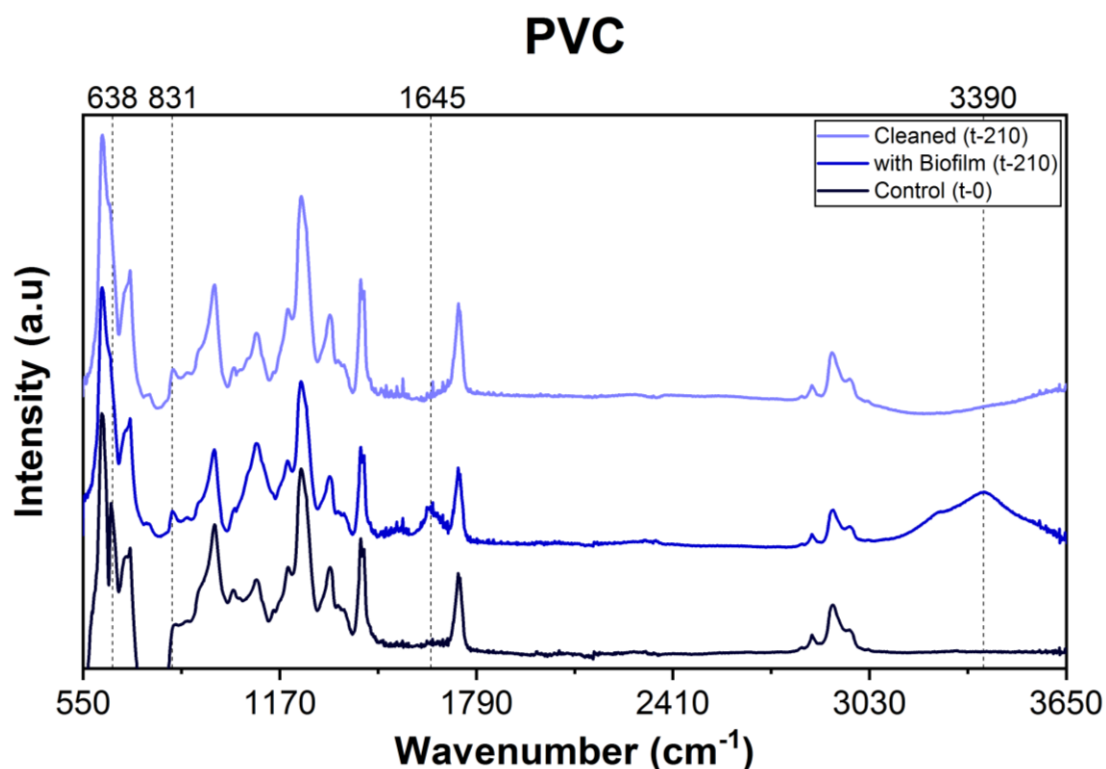


Figure 5.3.7 ATR-FTIR spectra of PVC films. Comparative spectra of PVC before (t-0) and after (t-210) days (with biofilm and cleaned) of the respirometric test. The intensity of peaks recorded as arbitrary units (a.u.).

For control PVC, the characteristics peaks were observed at the wavenumbers of 610, 1240, 1331, 1427, 2910 and 2970  $\text{cm}^{-1}$  corresponding to C–Cl bond, C–H bending bond and C–H stretching (Figure 5.3.7). These peaks were discussed in detail in Chapter 4 (Section 4.3.5). After the biodegradation test, the ATR-FTIR spectra of PVC with biofilm revealed new peaks at 831, 1645 and 3390  $\text{cm}^{-1}$ , corresponding to vinylidene group (compounds with  $\text{C}=\text{CH}_2$  functional group), C=C bonds and OH functional group, respectively. In addition, the peak at 638  $\text{cm}^{-1}$  (assigned to C–Cl bond) disappeared after the respirometric experiment. Similarly, the disappearance of a peak at 638  $\text{cm}^{-1}$  and appearance of a new peak at the wavenumber of 831  $\text{cm}^{-1}$ , were also observed for the PVC spectra after washing off the biofilm. However, the ATR-FTIR spectra of cleaned PVC film did not show peaks at 1645 and 3390  $\text{cm}^{-1}$  (Figure 5.3.7).

Finally, carbonyl indices (CIs) of HDPE, PET and PVC were determined relative to peaks at 2915  $\text{cm}^{-1}$  (C–H stretching), 1410  $\text{cm}^{-1}$  (C=C stretching) and 1427  $\text{cm}^{-1}$  (C–H bond) respectively. The CI of HDPE decreased after the biodegradation test but the decrease was not significant (Student's t-tests showed  $p > 0.05$ ). In contrast, there was an increase in the carbonyl indices of both PET and PVC. A Student's t-test showed that the difference between the CIs of PET before and after the respirometric analysis was significantly ( $p < 0.001$ ) higher compared to CIs of PVC ( $p < 0.05$ ) (Figure 5.3.8).

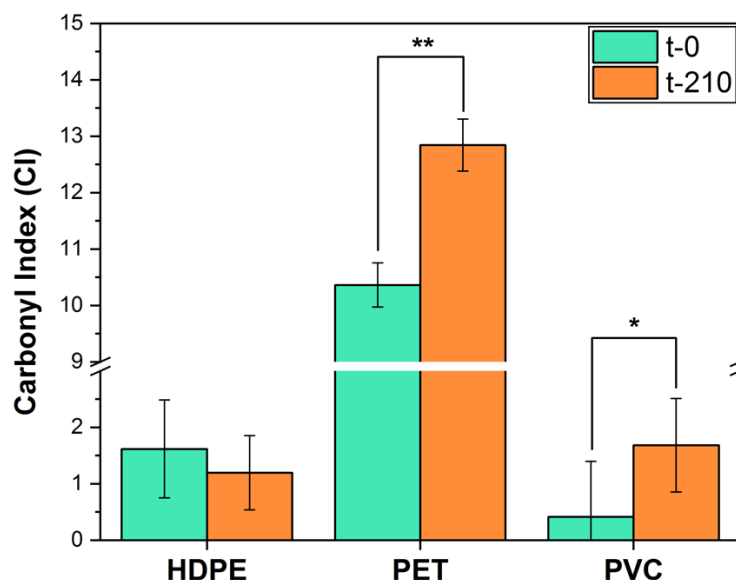


Figure 5.3.8 Carbonyl indices (CI) of HDPE, PET and PVC films before (t-0) and after (t-210) the biodegradation test. \*\*indicates significance  $p$ -value  $< 0.001$ , \*indicates significance  $p$ -value  $< 0.05$

## 5.4 Discussion

Marine bacterial consortia that could degrade three chemically distinct synthetic plastics were isolated using the enrichment, screening and isolation protocols employed in this Chapter (Section 5.2.2 and 5.2.3). A total of 19 bacterial isolates were isolated from the surface of all plastic-types. Taxonomic identification of isolates selected from HDPE film showed that they were closely related to the genera *Pseudoalteromonas*, *Rhodococcus*, *Oceanisphaera*, *Salinibacterium* and *Bacillus*. These genera predominantly contain marine and terrestrial bacteria (Holmström and Kjelleberg, 1999, Hayat et al., 2010, Elsayed et al., 2017). The enrichment of these genera in seawater with HDPE as a sole carbon source, could be due to their ability to degrade polyethylene and other synthetic plastics (Leathers et al., 2000, Kathiresan, 2003, Orr et al., 2004a, Sivan et al., 2006, Syranidou et al., 2017). In addition, several species of *Rhodococcus* are also known to be producers of a number of extracellular enzymes and have reported biodegradation of recalcitrant organic pollutants (Laczi et al., 2015, Yano et al., 2015).

From the PET film, the isolates belonged to the genera: *Salinibacterium*; *Oceanisphaera*; *Vibrio*; *Pseudoalteromonas* and a couple of closely related strains of *Bacillus* species, were isolated. Besides their previously described ability to degrade polyethylene, *Pseudoalteromonas* and *Bacillus* species have also shown the capability to degrade highly recalcitrant polycyclic aromatic hydrocarbons (Hedlund and Staley, 2006, Das and Mukherjee, 2007, Papa et al., 2009, Lily et al., 2009). Similarly, an *Oceanisphaera* strain has recently been reported to have biodegradation potential for a common environmental



pollutant, nicosulfuron (Zhou et al., 2017). The ability of these isolates to degrade complex aromatic compound molecules explains their enrichment in the seawater with PET, as the polymer chains possess a relatively high density of aromatic groups and therefore could be useful in PET biodegradation.

The bacteria isolated from the microbial growth on the PVC film (Figure 5.3.1) were identified as members of the genera *Bacillus*, *Rhodococcus*, *Salinibacterium*, *Vibrio*, *Epibacterium*, *Cellulophaga*, *Pseudomonas* and *Microbacterium*. The ability to degrade PVC by various strains of *Bacillus*, *Rhodococcus*, *Vibrio* and *Pseudomonas* is widely known (Danko et al., 2004, Danko et al., 2006, Sah et al., 2011, Das et al., 2012, Raghul et al., 2014, Yang et al., 2018, Kumari et al., 2019, Giacomucci et al., 2019). No scientific literature can be found to suggest the direct biodegradation of PVC by *Epibacterium*, *Cellulophaga*, and *Microbacterium* isolates. However, production of extracellular enzymes including; lipase, protease and depolymerase, have been reported previously for closely related strains of *Microbacterium* (Osman et al., 2015) and, therefore, might be able to biodegrade PVC, as the polymer is susceptible to depolymerase (Shah et al., 2008).

The selected isolates were used as a separate consortium to test their biodegradation potential for the respective plastic-type in a respirometric system. After incubation, the PVC vessels showed the highest mineralisation of 16.26 % whereas HDPE and PET achieved 8.71 and 6.51 %, respectively. There are no peer-reviewed published experiments to directly compare these results to, except for the study by Alvarez-Zeferino et al. (2015). They used sodium hydroxide-CO<sub>2</sub> trap respirometer system and reported mineralization of approximately 2.06 % for polyethylene using seawater as inoculum. In contrast to the current study, low degree of biodegradation has been reported previously in other environments for PET and HDPE. For example, 2.7 % biodegradation was reported for PET after incubation in composting conditions at 58 °C after 63 days (Kijchavengkul et al., 2006). Similarly, in a composting process inoculated with *Rhodococcus rhodochrous* HDPE biodegraded by 6 %. Soil incubations experiments exhibit similar results, for example, Fontanella et al. (2010) measured < 5% HDPE mineralization after 352 days of experimentation.

All of these studies have used a general microbial community, however, the higher rate of mineralization achieved for PVC, HDPE and PET in this study suggest degradation can only occur in the presence of selected microorganisms. These microorganisms can be isolated after enriching in their respective plastics or found exclusively in environments submitted to continuous contamination by the polymer (Chiellini et al., 1999). Similarly, HDPE and PET

degrading microorganism were isolated from their respective polluted environments (Balasubramanian et al., 2010, Yoshida et al., 2016). The selective pressure exerted by the acclimation procedure tends to further increase the population of these plastic degrading microorganisms. Moreover, the enrichment of the selected microorganisms could also explain the low degree of cellulose assimilation by these microbes, as revealed by the very limited biodegradation (3.74 %) of the cellulose sample (Figure 5.3.3). In contrast, the cellulose is highly biodegradable material and it has the >70 % of degradation rate in respirometric systems (Kijchavengkul et al., 2006, Way et al., 2010, Bettas Ardisson et al., 2014).

Physicochemical properties of the plastics were analysed by SEM and ATR-FTIR spectroscopy to assess their degradation after the respirometric test. The surface morphology of all the tested plastics changed after their incubation with corresponding bacterial consortia which further support the biodegradation of the plastics. Surface textures such as fissures and cracks appeared on HDPE and the surface became irregular, whereas PET and PVC films displayed pits. These morphological changes were attributed to the formation of the microbial biofilm on the plastic surfaces, indicating the tendency of bacteria towards the plastics in order to utilise it as carbon and energy source. Such alteration of the plastic surfaces due to microbial growth has been elucidated by many studies (Raghul et al., 2014, Restrepo-Flórez et al., 2014, Das and Kumar, 2015), where grooves and pits were detected superficially after polymers were subjected to biodegradation.

The morphological changes were substantiated by ATR-FTIR analysis revealing changes in the chemical composition of plastics due to microbial activity. After the biodegradation test, all the plastic materials with attached biofilms shared one common OH functional group peak at  $3390\text{ cm}^{-1}$ . This could either be due to the presence of water in the attached microbial cells (Nichols et al., 1985) or the presence of salts in the seawater (Peters and Ewing, 1997). In addition, a recent study on environmental degradation of polyethylene in seawater reported that the peak was due to the formation of a new functional group and was not disappeared after washing the plastic (Da Costa et al., 2018). However, the subsequent washing of plastic films in the current study revealed that the peak disappeared in the corresponding spectra, thus confirming the earlier peak due to the presence of either water in the attached microbial cells.

Distinct new peaks were also detected on each plastic-type in comparison to their untreated controls. For HDPE, new peaks appeared at  $1120$  and  $1186\text{ cm}^{-1}$ , suggesting the presence of



alkoxy and acyl C–O stretching bands. These bands are the indicator of alcohol, ether and ester formation by microbial activity and could be formed due to oxidation of HDPE (Jeon and Kim, 2013), thus confirming the HDPE degradation. Similar peaks were observed in previous studies examining the extent of polyethylene degradation in marine environments (Syranidou et al., 2017, Da Costa et al., 2018).

For PET, two new peaks were detected at 1576 and 2970  $\text{cm}^{-1}$  corresponding to C=C and C–H bond, respectively. Though new double bonds could be formed due to microbial hydrolysis of ester bond at 1712  $\text{cm}^{-1}$  (Webb et al., 2013) and could result in the formation of peaks in the region of 1615– 1570  $\text{cm}^{-1}$ . However, no change was observed in the peak intensity of ester bond at 1712  $\text{cm}^{-1}$ , suggesting that the C=C peak appeared at 1576  $\text{cm}^{-1}$  was due to the structural aromatic ring of PET and not because of the formation of new double bonds. In contrast, the formation of alkane bond (C–H) could be due to the degradation of PET as a similar bond formation was reported for PET degrading in the marine environment (Ioakeimidis et al., 2016)

The PVC spectra presented a new peak at 831  $\text{cm}^{-1}$ , corresponding to the functional group of vinylidene (C=CH<sub>2</sub>). This group is usually formed by the cross-linking of smaller PVC chains after successive chain scission reactions (Allothman et al., 2012), which results from oxidation of PVC (Shi et al., 2008). Since microbes are known to produce enzymes which can catalyse the oxidative depolymerization of plastics (Kırbaş et al., 1999), this suggests that the oxidation and subsequent degradation of PVC by the microbial consortium used in this study. In addition, the peak at 638  $\text{cm}^{-1}$  (assigned to C–Cl bond) disappeared after the respirometric experiment. This could be due to the dechlorination of PVC which is an abiotic degradation phenomenon resulting from UV radiation (Gewert et al., 2015). Since the biodegradation test was not performed in the dark, it is possible that photo-induced dechlorination of PVC might have occurred in the vessels.

The spectra of all plastics were then investigated to measure the carbonyl index; a commonly used indicator to characterise biodegradation by determining the oxidation of plastic materials. The carbonyl index of the HDPE slightly decreased was its control, which could be explained by the mineralization of the degradation products by microorganisms. A similar decrease in the carbonyl index of polyethylene has been reported by previous studies due to bioassimilation of oxidised PE in biologically active environments (Chiellini et al., 2003, Hadad et al., 2005, Sudhakar et al., 2008, Syranidou et al., 2017). In contrast, the carbonyl index of PET and PVC were significantly increased which could be due to slow

biodegradation of these polymers as they are resistant to microbial degradation (Gewert et al., 2015, Wei and Zimmermann, 2017). PET is also susceptible to hydrolysis which leads to a decrease in molecular weight and an increase in carboxylic acid end groups (Gewert et al., 2015), therefore, explaining the increase in carbonyl index.

## **5.5 Conclusions**

This study aimed to demonstrate the biodegradation potential of marine microbes selected for synthetic polymers in a marine environment. The first part of the study concerned with the isolation of mixed bacterial isolates having enhanced biodegradation ability. The enriched bacterial community showed growth on the respective plastics and selected for further biodegradation test. In the second part of the study, the biodegradation of the plastics was examined using respirometric method under simulated seawater conditions. PVC was found to biodegrade with 16.26 % mineralisation after incubation for 210 days, whereas HDPE and PET achieved 8.71 and 6.51 % of mineralisation, respectively. The biodegradation was further confirmed by SEM images with significant surface alterations and chemical changes were elucidated by ATR-FTIR. The results suggest that the microbial degradation of these plastics can only occur in the presence of selected microorganisms and the selective pressure exerted by the enrichment method tends to increase the population of these plastic degrading microorganisms. Furthermore, the role of the selected bacterial isolates, in terms of their specific functions and metabolic activities associated with biodegradation, is not yet completely characterized. Therefore, further experiments are required in order to better understand and predict the mechanisms and kinetics of the synthetic plastics biodegradation in natural marine environments.

## Chapter 6: General Discussion

This thesis aimed to study the effects of plastic pollution on the microbial and chemical properties of seawater and the role microorganisms played in the early stages of plastic decomposition. Initially, a short-term laboratory microcosm experiment was carried out to evaluate the potential impacts of seven different plastics on seawater bacterial communities. The structure, diversity and taxonomic identities of bacterial communities were determined by T-RFLP and amplicon sequencing (Chapter 2). Temporal biofilm formation on three chemically distinct plastics was then investigated within natural seawater in a long-term exposure experiment. The biofilms were characterised and visually confirmed using amplicon sequencing and SEM, respectively (Chapter 3). Additionally, physical and chemical degradation of the exposed plastics were quantified by SEM, contact angle, tensile testing and FTIR spectroscopy methods (Chapter 4). Finally, marine consortia were developed using an enrichment method to determine their ability to biodegrade selected plastics in a marine environment. The ultimate level of biodegradation was quantified using a respirometric system and then visually and chemically confirmed by SEM and FTIR spectroscopy (Chapter 5). In this Chapter, key findings of the thesis are discussed and summarised, and recommendations for future work are made.

### 6.1 Effect of Plastics on Marine Microorganisms

Chapter 2 described the potential effects of synthetic and biodegradable plastics on seawater microbial communities. The results showed that when plastics were introduced into seawater, they changed the inherent chemical and biological properties, and induced major shifts in the composition and diversity of the microbial community in the water. Two of the most important drivers of microbiological shifts in seawater were changes in DOC concentration and pH caused by the presence of plastic. Moderate changes in pH have been previously shown to cause shifts in bacterial communities when measuring the effects of ocean acidification as a result of elevated CO<sub>2</sub> levels in the atmosphere (Krause et al., 2012). Furthermore, based on the results of this thesis, the presence of plastic pollution in the world's ocean may contribute to localised increases in ocean acidification. A dilution effect may mitigate this to some extent but may have implications for ocean chemistry. This could, for example, indirectly affect organisms that incorporate calcium carbonate into their shells, e.g.

corals (Doney et al., 2009). Corals may be particularly affected because plastic degradation in the oceans will likely lead to an increase in DOC levels as well. Elevated DOC levels have been shown to accelerate the growth rate of microbes living on coral surfaces by an order of magnitude, which leads to mortality because of a disruption in the balance between the coral and its associated microbiota (David et al., 2006).

Although DOC was leached from all plastic types used for this study, the highest concentrations of DOC were leached from biodegradable plastics. In both cases, the DOC leaching from the plastics may contribute to the oceanic DOC which supports micro-heterotrophic growth (Hansell et al., 2009, Romera-Castillo et al., 2016) and plays an important role in carbon cycling by acting as a vector for quick transfer of carbon and energy in food webs (Kirchman et al., 1991). This is a significant novel finding of this thesis that furthers the understanding/consequences of plastic pollution in the world's oceans. Recently, Romera-Castillo et al. (2018) estimated that up to 236,000 MT of such DOC could be leached from the synthetic plastics per year in the global ocean and could make up to 10 % of the oceanic DOC in the area of high plastic contamination. However, based on the findings of this thesis, it is plausible that leached DOC could be much higher than these estimates due to higher amounts of DOC released from biodegradable plastics. This may increase in future as the nature of plastic pollution will evolve with the advent of new biodegradable plastics, for example, global production of biodegradable plastics is currently 2.11 million tonnes and expected to increase by 20 % to 2.62 million tonnes in 2023 (European Bioplastics, 2018).

An increase in oceanic DOC that supports heterotrophic bacterial activity, may subsequently lead to increased competition for inorganic nutrients between bacteria and phytoplankton (Thingstad et al., 2008). This may weaken the link between phytoplankton production and bacterial activity and expand the zones of the net heterotrophy (Traving et al., 2017b). These zones have higher concentrations of CO<sub>2</sub> in the seawater than in the atmosphere above and the excess CO<sub>2</sub> is released to the atmosphere (Traving et al., 2017b). This may have consequences for global carbon cycling as it may change the oceans from a net CO<sub>2</sub> sink to a net CO<sub>2</sub> source. This research, therefore, raises further questions beyond the scope of the original work and suggests that fossil fuel derived and biodegradable plastics could have unintended consequences when they breakdown in the natural environment.

The bioavailability of the leached DOC stimulated microbial growth which causes a decrease in the pH of the seawater. The distinct patterns of DOC bioavailability appeared to induce shifts in the composition of bacterial communities when analysed by 16S rRNA gene T-

RFLP profiling. For synthetic plastics, the bioavailable DOC decreased following an initial increase which could be due to re-adsorption of DOC onto the plastics, as has been previously reported (Romera-Castillo et al., 2018), or due to utilisation by heterotrophic microorganisms. The availability of DOC explains the distinct compositional shifts in synthetic plastic treatments. In contrast, with the biodegradable plastic treatment, the concentration of bio-available DOC continued to increase over time, which could be due to water-soluble products generated by biodegradation of these plastics (Sakai et al., 1998, Matsumura et al., 1999, Kasuya et al., 2000). Therefore, a clearer shift in the bacterial communities were observed with the biodegradable plastics, due to the bioavailability of DOC. This contrasting result for synthetic and biodegradable plastic treatments highlights the high temporal variability of leached DOC which should be considered when examining the long-term effects of plastics on marine microorganisms.

Following the taxonomic identification of TRFs through amplicon sequencing data, it was revealed that the bacterial communities differed in their response to the substrates released from the plastics, and consequently, certain bacterial communities were selected. The bacterial groups belonging to the classes *Alphaproteobacteria*, *Gammaproteobacteria* and *Bacteroidetes* proliferated most within all plastic treatments and only differed in terms of their relative abundances. The proliferation of these bacterial groups suggests potential degradation and assimilation of complex carbon substrates, including leached DOC (Carvalho et al., 2006, Le Borgne et al., 2008, Kostka et al., 2011, Gutierrez et al., 2014, Sipler et al., 2017). It is well established that bacterioplankton communities have the ability to respond to environmental disturbances (Langenheder et al., 2005, Allison and Martiny, 2008, Comte and del Giorgio, 2011, Sjöstedt et al., 2012) and can either become sensitive (i.e., decrease in relative abundance), resistant (i.e., maintain relative abundance), or responsive (i.e., increase in relative abundance) (Allison and Martiny, 2008). Based on the findings of this thesis, the bacterioplankton communities can be considered both sensitive and responsive to the presence of plastics, with the different response of individual bacterial populations suggesting the presence of plastics in oceans will have the potential to cause profound changes in bacterioplankton community composition. Like other environmental perturbations, this may also have implications for many properties of bacterioplankton community (e.g. bacterial production and activity) that heavily influence ecosystem functioning by changing the flow of carbon (Comte and del Giorgio, 2011, Lindh et al., 2015).

It is also important to note that the observed changes among bacterial populations in Chapter 2 are the result of adaptation in a closed system. The distribution of bacterial populations in the natural marine environment is limited by very few physical barriers and therefore dispersal is likely an important factor for bacterioplankton responses to plastic pollution. Nevertheless, these observations highlight substantial effects of plastic-induced shifts in local environmental conditions for regulating bacterioplankton community composition and provides a starting point for research into the ecology and functions of microbial assemblages in areas with high plastic waste concentrations. Where plastic pollution is concentrated in the world's oceans (e.g. the gyres), it could be that over time, this represents a new micro-scale environmental gradient, where physicochemical parameters are altered due to the presence of plastic pollution and therefore a habitat heterogeneity along a gradient is created which results in a unique niche for microorganisms (Dang et al., 2019).

## **6.2 Marine Biofilm Formation on Plastics**

In Chapter 3, microbial colonisation on three chemically distinct synthetic polymers was analysed within a long-term (56 weeks) exposure experiment, with the characteristic plastic-attached communities studied to assess and identify patterns of biofilm temporal change. Microbes were found to colonise all plastic-types, exhibiting a successional increase with the sampling interval. Both the structure and diversity of the plastic-associated assemblages were significantly different from those found within surrounding seawater. Zettler et al. (2013) coined the term “Plastisphere” for these plastic colonising microorganisms, which are based on the difference between the microbial communities present on the plastic materials and their planktonic counterparts. Similarly, the uniqueness of plastic-associated assemblages was confirmed in several later studies (Oberbeckmann et al., 2014, Amaral-Zettler et al., 2015, De Tender et al., 2015, Bryant et al., 2016, Debroas et al., 2017, De Tender et al., 2017a, Jiang et al., 2018, Kirstein et al., 2018). Therefore, the results of this thesis (Chapter 3) and the findings of previous studies point towards the consensus that Plastisphere communities are different from those living freely in seawater. Since it is well recognised that the surface chemodynamics (e.g. surface conditioning and nutrient enrichment) play a role in forming distinct biofilm communities (Dang and Lovell, 2016), the uniqueness of the Plastisphere communities is likely due to specific surface conditioning. Based on the findings of Chapter 2, there was evidence to suggest that the DOC leached from plastics was re-adsorbed onto their surfaces, therefore, further conditioning the surface for the subsequent attachment of unique bacterial taxa that were likely to use DOC as a

carbon source. This may have fostered the development of unique Plastisphere communities on the tested plastics. The plastic-associated bacterial communities were analysed for their specificity. The results indicated that the Plastisphere communities were significantly different from each other. This was contrary to the results from previous studies which investigated the biofilm formation over a short incubation period and did not detect significant differences between distinct plastic types (Oberbeckmann et al., 2016, Oberbeckmann et al., 2018). The discrepancy between these observations and the differences between the Plastisphere communities, detected in Chapter 3, is surprising since it has been demonstrated that bacterial communities present on dissimilar surfaces evolve to a similar community structure over time (Salta et al., 2013, De Tender et al., 2017a). However, a recent long-term study on nine distinct plastics observed significant differences in the composition of biofilm communities (Kirstein et al., 2018). Based on these findings and the insights gained in Chapter 3, it is possible that a longer exposure to seawater may have introduced unique bacteria to the biofilm communities, which subsequently resulted in a significant difference amongst the communities. Moreover, the physicochemical factors of plastics, which includes: colour; absorbed chemical; roughness; vulnerability to weather; surface conditioning and nutrient enrichment may have also played a role in forming distinct biofilm communities (De Tender et al., 2015, Dang and Lovell, 2016, Frère et al., 2018, Parrish and Fahrenfeld, 2019). These factors were not studied in this thesis; therefore, future work could include these parameters while investigating the specificity of the Plastisphere communities in long-term experiments.

Comparison of the bacterial Plastisphere communities revealed a common core bacteriome on all the plastics, which could be divided into three groups based on the timing of their peak relative abundance. Despite a number of studies detailing Plastisphere microbial communities in the world's oceans, there are only two studies that describe microbial communities attached to plastic surfaces close to the UK (North Sea) (De Tender et al., 2017a, Kirstein et al., 2018). Comparison with these studies showed similarities at phylum, class and family level, however, no similarities were found at genera level except for *Nitrospira*. The dissimilarities between these biofilm communities could be attributed to the difference in the composition of bacterial taxa of contrasting water bodies (De Tender et al., 2015, De Tender et al., 2017a) as the current study was conducted in the English Channel. In addition, localised and regional differences in environmental factors (e.g. hydrodynamics, temperature, water chemistry and nutrient availability) can also influence the composition of biofilm communities (Salta et al., 2013). This, therefore, suggests that the source communities within the surrounding water and the inherent specific environmental

conditions shaped the colonisation patterns on plastic surfaces in this study. These findings agree with previous studies (Oberbeckmann et al., 2014, Amaral-Zettler et al., 2015, Oberbeckmann et al., 2016, Kirstein et al., 2018), showing that geographical location and seasonal variations drive the Plastisphere communities in marine habitats. It remains unclear however whether core microorganisms are selected by the hard surface of plastic or by the chemical composition of the plastic itself. The latter may be an important factor, as the results from this thesis show that some of the microbes among the core bacterial community are plastic specific, therefore, they may be used as indicator organisms that are preferentially able to interact with plastics and use it as potential carbon source in marine environments.

### **6.3 Physical Degradation of Plastics**

Textural investigations were carried out in parallel to the microbial analyses. The results indicated that tested plastics, exposed in seawater, exhibited many of the same surface textural characteristics as previously reported plastics from beaches and the world's oceans (Corcoran et al., 2009, Cooper and Corcoran, 2010, Zbyszewski and Corcoran, 2011, Fotopoulou and Karapanagioti, 2015, Cai et al., 2018, Da Costa et al., 2018, Tang et al., 2018). The gradual development of uneven surfaces for all the tested plastics suggested delamination of the upper layers and formulation of small pieces (microplastics). The ecological impacts of microplastics are well recognised (Andrady, 2011, Andrady, 2017), and pose greater environmental risks than the original intact items of plastic waste (Browne et al., 2007, Wright et al., 2013b, Wu et al., 2019).

In this study, the overall degradation rate (as measured by tensile stress test) was found to be slow for all the tested plastics. This could be attributed to lower temperatures and attenuated UV light in seawater. In addition, the colonising microorganisms on the plastic films (Chapter 3) may further reduce the UV irradiation by covering the surface and shielding the plastic from UV light (O'Brine and Thompson, 2010, Welden and Cowie, 2017). It is interesting to note that the biofilm attached to plastics did not contribute to the plastic degradation in seawater, however, there was evidence to suggest that some members of the biofilm community (e.g. *Pseudomonas* and *Cyanobacteria*) had the potential to biodegrade plastics (Chapter 3, Section 3.4). Chemical degradation characteristics were also less prominent on plastic samples which may be a result of less time at sea along with the environmental conditions. No significant difference between the seawater exposed and control samples suggests that the degradation rate is slow and is in accordance with previous studies (Pegram and Andrady, 1989, Andrady, 1990, Andrady et al., 1993a, Biber et al.,



2019). This is an expected finding as plastics submerged in seawater, which make up 60 % of debris (Kukulka et al., 2012), degrade much slower due to biofilm formation. The biofilm shields the plastics from UV light and makes the plastic negatively buoyant and sink (Fazey and Ryan, 2016, Weinstein et al., 2016). As a result, the exposure to light, shear stress, oxygen and temperature will be limited and therefore reduce degradation (Gewert et al., 2015). However, hydrolytic degradation of plastics, particularly for PET, is also possible because of the exoenzymes produced by microorganisms within the marine environment (Gewert et al., 2015, Bollinger et al., 2020). Such hydrolytic degradation reactions preferentially occur at the amorphous regions of the polymer (Donelli et al., 2009, Herrero Acero et al., 2011).

The data in Chapter 4 showed that synthetic plastic formulations persist and do not degrade quickly in a long-term (i.e., > 1 year) exposure to natural seawater. In addition, the experiment undertaken provided a more realistic proxy for the degradation process, as the test materials experienced open ocean conditions rather laboratory controlled exposures that often use idealised test conditions (Brandon et al., 2016, Da Costa et al., 2018, Cai et al., 2018, Tang et al., 2018). From the perspective of marine litter, the current study was limited by the selection of plastics used, which can only be considered as representatives of a few types within the wide range of plastics usually found in marine debris (Bond et al., 2018). Different formulations of each plastic might respond to environmental exposure in different ways. The plastics used for this study were selected because they represented 38 % of global polymer production (Geyer et al., 2017) and they were also widely reported as major components of plastic debris in the world's oceans (Barnes et al., 2009, Morét-Ferguson et al., 2010, Browne et al., 2011, Duhec et al., 2015). Therefore, the patterns, similarities and differences highlighted here in terms of their degradation in natural seawater, should stimulate further, more detailed investigations in the future.

## 6.4 Biodegradation of Plastics

Chapter 5 described the isolation and assessment of marine bacterial consortia with the potential to biodegrade chemically distinct synthetic plastics. The bacterial consortia were dominated by members of *Actinobacteria*, *Alpha-*, *Gamma-proteobacteria*, *Flavobacteriia* and *Firmicutes*. Members of these taxonomic classes were also identified as most responsive to plastic additions (Chapter 2) and plastic surface colonisers (Chapter 3). The isolates selected for biodegradation of tested plastics were the nearest taxonomic relatives of *Pseudoalteromonas*, *Oceanisphaera*, *Rhodococcus*, *Salinibacterium*, *Bacillus*, *Vibrio*,

*Epibacterium*, *Cellulophaga*, *Pseudomonas* and *Microbacterium* species. Prior literature also suggested that closely related strains of the selected isolated have the ability to degrade recalcitrant materials (Hedlund and Staley, 2006, Das and Mukherjee, 2007, Papa et al., 2009, Lily et al., 2009).

The biodegradability test revealed that the consortium selected for PVC had the highest biodegradation potential compared to HDPE and PET with their respective consortia. This suggests that the microbial degradation of these plastics can only occur in the presence of selected microorganisms after enrichment and isolation, or microorganisms found exclusively in environments submitted to continuous contamination by the polymer (Chiellini et al., 1999, Yoshida et al., 2016). There is a need for more enrichment strategies to isolate plastic degrading microorganisms, as cultivation dependent work will enhance our ability to perform further cultivation independent assessments and will give the insight into the metabolic capabilities of these versatile microorganisms.

Although ASTM D 6691-09 (ASTM-International, 2009) test used in this thesis is the standard test method to quantify the degree of plastic biodegradation in the aerobic marine environment, this cannot be considered as proof of ultimate biodegradation, but rather an indication about a potential for biodegradation in the oceans (Jacquin et al., 2019). Therefore, this test cannot be used to realistically predict the biodegradability in the world's oceans, therefore the results presented are not representative of *in situ* rates of plastic biodegradation. Various limitations can be identified that should be taken into account when interpreting this Chapters results. These include: (1) underestimation of biodegradability time, (2) use of enriched consortia and (3) temperature used. Firstly, this test significantly underestimated the time required for polymer biodegradation within the marine environment. According to test guidelines, the maximum test duration should be six months, but the CO<sub>2</sub> evolutions did not reach a plateau during the experiment, which suggests that total conversion of plastic into biomass and CO<sub>2</sub> did not occur. Secondly, using preselected or preconditioned strains is not a true representation of the world's oceans and represents an idealistic approach which means the results cannot be representative of the natural environment. These uncertainties are well recognised in the scientific literature (Muller, 2005, Harrison et al., 2018a). Finally, the test temperature was higher than those encountered within the natural environment and did not account for seasonal fluctuations in conditions which could influence the composition and activities of plastic-associated microorganisms (Sudhakar et al., 2008, Artham et al., 2009a, Oberbeckmann et al., 2014).

## 6.5 Recommendations for future work

Results from this thesis shed light on the impacts of plastics on marine microbial communities, their colonisation and extent of degradation in the marine environment. There does, however, remain scope for further work as detailed below. These studies should include:

- In Chapter 2, plastics appeared to affect the bacterial communities in seawater and diverse changes in composition and diversity were observed. However, it remained unclear that the resultant community change had an effect on its functional performance as this study did not measure the metabolic activities of the bacterial communities, which can be elucidated by mRNA assays (Moran et al., 2013).
- The impacts of plastics were only explored for bacterial communities, however, eukaryotes, especially fungi which play a major role in marine ecosystems as decomposers (Hyde et al., 1998) were not studied. Therefore, this should be addressed in future studies.
- In Chapter 3, the plastic attached bacterial communities were investigated but the SEM images showed the attachment of various eukaryotes (e.g. diatoms, dinoflagellates and fungi). Therefore, future studies should examine the complete Plastisphere communities including eukaryotes in similar short time intervals as used for this thesis, to understand the influence of eukaryotes in the biofilm development and recruitment of specific species on the biofilm.
- Since it is well established that environmental factors influence biofilm communities (Salta et al., 2013) therefore, Plastisphere communities should be monitored at close time intervals over more than one seasonal cycle, and at different locations to delineate the effects of season and habitat variation.
- In Chapter 4, the degradation of plastics was only monitored in submerged condition, however, plastics also occur on the surface and in intertidal habitats. Therefore, future studies should also include these habitats for better understanding of the fate of plastics in open oceans.
- In Chapter 5, various marine bacterial isolates were selected based on their growth on plastics and were subsequently used for a respirometric investigation to determine their ability to biodegrade plastics. Another method to actually indicate the degradation of plastic is by studying enzyme production by microbial strains (Yoshida et al., 2016). Therefore, future studies should study the enzyme production by these isolated strains.

## 6.6 Conclusions

Based on the findings of Chapter 2 following conclusions were drawn:

- The presence of plastics induced major shifts in the composition and diversity of the microbial community in the water.
- Two of the most important drivers of microbiological shifts in seawater were changes in DOC concentration and pH caused by the presence of plastic.
- DOC was leached from all the plastics; however, the highest concentrations of DOC were leached from biodegradable plastics.
- The bacterial groups belonging to the classes *Alphaproteobacteria*, *Gammaproteobacteria* and *Bacteroidetes* proliferated most within all plastic treatments and only differed in terms of their relative abundances.
- The change in community was significantly correlated with the concentrations of DOC, TN and the pH change.

Based on the findings of Chapter 3 following conclusions were drawn:

- The Plastisphere communities are different from those living freely in seawater.
- The results indicated that the Plastisphere communities were significantly different from each other.
- Comparison of the bacterial Plastisphere communities revealed a common core bacteriome on all the plastics, which could be divided into three groups based on the timing of their peak relative abundance.
- Despite the similarities among HDPE, PET and PVC associated bacterial communities, a few distinctively unique OTUs were observed for all the three plastic attached communities across the sampling points. This indicated that physicochemical factors of plastics shape the biofilm communities in addition to the surrounding seawater.

Based on the findings of Chapter 5 following conclusions were drawn:

- It was concluded that the surface properties of plastic material changed significantly over time when submerged in seawater. This was attributed to the attachment of microorganisms
- The surface wettability of all tested plastics was also changed significantly in this study

- The changes in tensile stress differed among the tested plastics. The tensile stress of HDPE decreased slowly but significantly over time, indicating that HDPE film demonstrated degradation in seawater. However, no significant loss in tensile stress was observed for PET and PVC.
- Chemical degradation characteristics were also less prominent on plastic samples which were attributed to attenuated UV, lower average temperature and biofilm formation of plastic surfaces.

Based on the findings of Chapter 6 following conclusions were drawn:

- Marine bacterial consortia that could degrade three chemically distinct synthetic plastics were isolated using the enrichment, screening and isolation protocols
- The biodegradability test revealed that the consortium selected for PVC had the highest (16.26 %) biodegradation potential compared to HDPE (8.71 %) and PET (6.51 %) with their respective consortia.
- Although the test used in this thesis is the standard test method to quantify the degree of plastic biodegradation in the aerobic marine environment, this cannot be considered as proof of ultimate biodegradation, but rather an indication about a potential for biodegradation in the oceans.

## Appendices

### SIMPER analysis of bacterial communities

Table. Appendix A.1 SIMPER analysis of bacterial communities, presenting the total similarity within all sampling points and dissimilarity between the control and plastics treated communities. Av.Si%: average percentage similarity within all sampling points, Av.δi %: average dissimilarity between the control and plastics treated bacterial communities.

Av.Si%		Av.δi%							
Control	88.36	Control	PET	HDPE	LDPE	PVC	PVOH	PLA	
PET	78.93	PET	37.06						
HDPE	80.52	HDPE	30.45	33.23					
LDPE	84.12	LDPE	32.6	36.42	30.73				
PVC	77.69	PVC	36.45	36.95	35.44	37.08			
PVOH	81.15	PVOH	35.42	39.66	38.62	39.49	37.71		
PLA	77.28	PLA	35.3	37.49	36.52	36.93	37.78	37.26	
PHB/PHV	75.39	PHB/PHV	41.84	47.86	44.25	45.28	43.56	42.33	44.56

## PERMANOVA pair-wise tests

Table. Appendix B.1 PERMANOVA pair-wise tests of bacterial communities within plastic treatments and control based on Bray-Curtis similarities index. Significance results ( $p(\text{perm}) < 0.05$ ) are in bold

	Comparison	t	p(perm)	Unique perms
Control vs	PET	1.4197	0.052	998
	HDPE	1.7335	<b>0.015</b>	998
	LDPE	2.4349	<b>0.001</b>	998
	PVC	1.9151	<b>0.004</b>	999
	PVOH	2.2739	<b>0.001</b>	999
	PLA	1.4909	<b>0.03</b>	999
	PHV/PHB	2.5034	<b>0.001</b>	999
PET vs	HDPE	1.6228	<b>0.014</b>	999
	LDPE	2.2914	<b>0.001</b>	999
	PVC	1.7025	<b>0.012</b>	998
	PVOH	2.1629	<b>0.002</b>	998
	PLA	1.764	<b>0.003</b>	999
	PHV/PHB	2.6316	<b>0.001</b>	999
HDPE vs	LDPE	2.3057	<b>0.002</b>	998
	PVC	2.2896	<b>0.001</b>	999
	PVOH	3.1376	<b>0.001</b>	999
	PLA	2.1012	<b>0.001</b>	999
	PHV/PHB	3.2663	<b>0.001</b>	999
LDPE vs	PVC	2.8059	<b>0.001</b>	998
	PVOH	3.2872	<b>0.001</b>	998
	PLA	2.5846	<b>0.001</b>	998
	PHV/PHB	3.5022	<b>0.001</b>	998
PVC vs	PVOH	2.0245	<b>0.001</b>	999
	PLA	1.7877	<b>0.003</b>	998
	PHV/PHB	2.5057	<b>0.001</b>	999
PVOH vs	PLA	1.8311	<b>0.003</b>	998
	PHV/PHB	2.3197	<b>0.001</b>	998
PLA vs	PHV/PHB	2.1599	<b>0.001</b>	998

## PERMDISP pair-wise tests

Table. Appendix C.1 PERMDISP pair-wise tests of bacterial communities within plastic treatments and control based on Bray-Curtis similarities index. Significance results ( $p$  (perm) < 0.05) are in bold

	Comparison	t	p(perm)
Control vs	PET	1.8645	0.091
	HDPE	0.005033	0.998
	LDPE	0.78666	0.477
	PVC	1.9908	0.093
	PVOH	2.0946	0.088
	PLA	2.7984	<b>0.022</b>
	PHV/PHB	3.722	<b>0.005</b>
PET vs	HDPE	1.9934	0.078
	LDPE	1.1247	0.283
	PVC	0.33629	0.777
	PVOH	0.42055	0.717
	PLA	1.38	0.215
	PHV/PHB	2.268	0.057
HDPE vs	LDPE	0.83627	0.437
	PVC	2.099	0.076
	PVOH	2.2125	0.059
	PLA	2.9155	<b>0.005</b>
	PHV/PHB	3.8991	<b>0.002</b>
LDPE vs	PVC	1.3301	0.225
	PVOH	1.4292	0.21
	PLA	2.2387	0.053
	PHV/PHB	3.1684	<b>0.007</b>
PVC vs	PVOH	0.071082	0.948
	PLA	1.007	0.386
	PHV/PHB	1.7964	0.134
PVOH vs	PLA	0.95464	0.456
	PHV/PHB	1.7512	0.139
PLA vs	PHV/PHB	0.66422	0.557



## Rarefaction curves of seawater bacterial communities

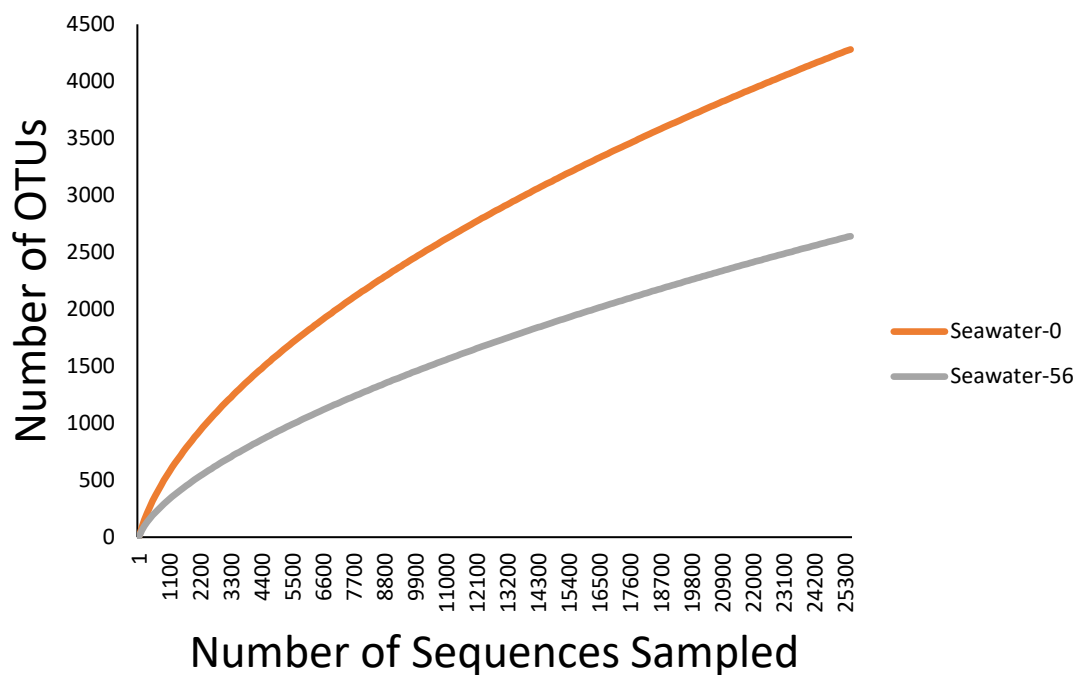


Figure. Appendix D.1 Rarefaction analyses, indicating the  $\alpha$ -diversity of bacterial communities in the seawater samples at the beginning (0-week) and end (56-week) of exposure experiment.

## Rarefaction curves of HDPE biofilm communities

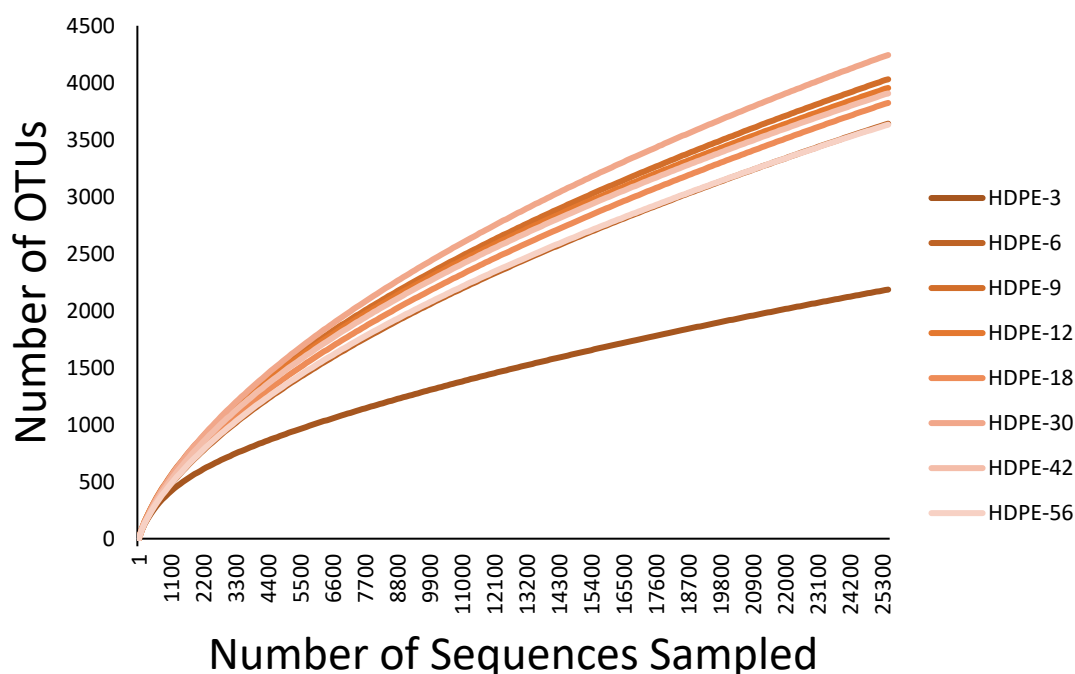


Figure. Appendix E.1 Rarefaction analyses, indicating the  $\alpha$ -diversity of HDPE biofilm bacterial communities at the sampling period of 3, 6, 9, 12, 18, 30, 42 and 56 weeks.

## Rarefaction curves of PET biofilm communities

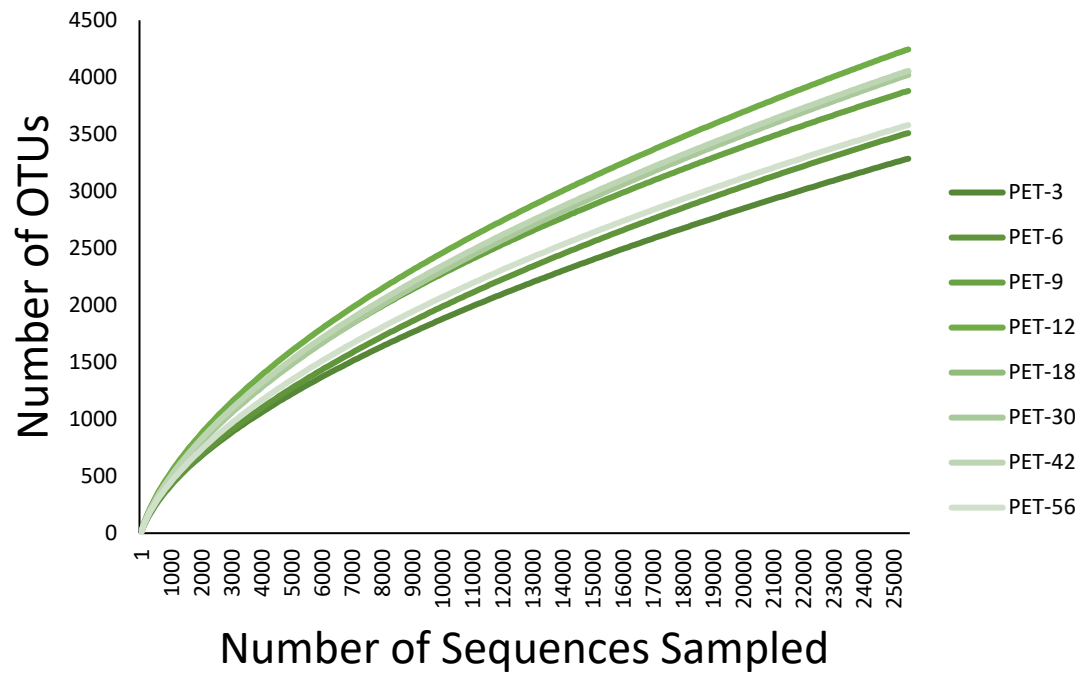


Figure. Appendix F.1 Rarefaction analyses, indicating the  $\alpha$ -diversity of PET biofilm bacterial communities at the sampling period of 3, 6, 9, 12, 18, 30, 42 and 56 weeks

## Rarefaction curves of PVC biofilm communities

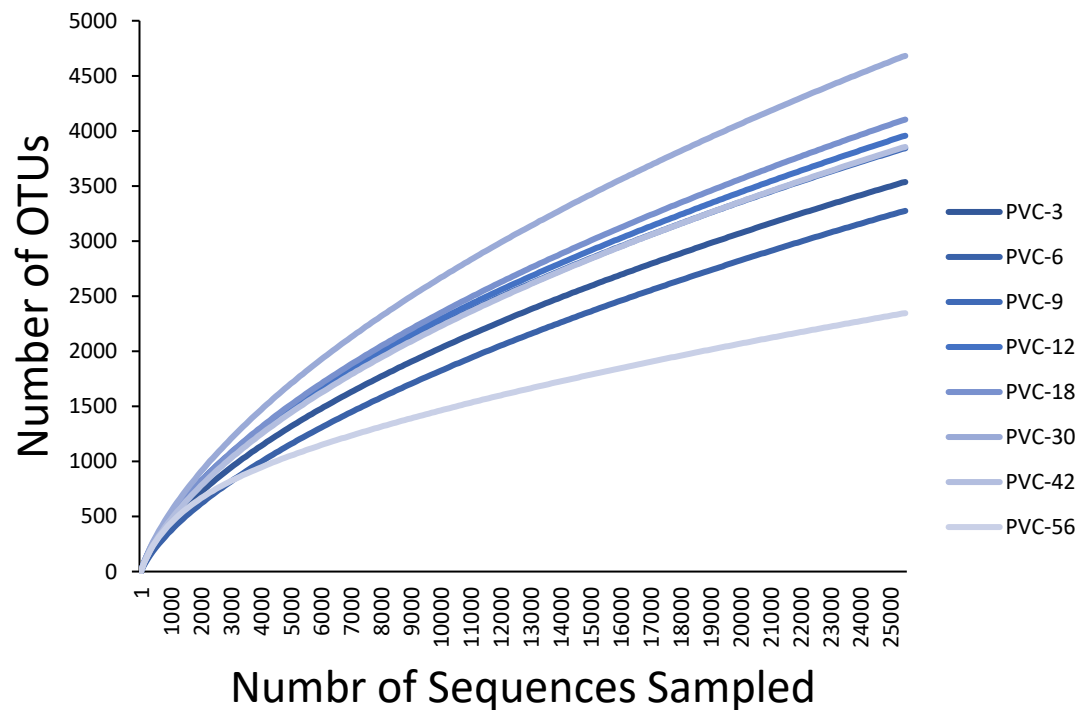


Figure. Appendix G.1 Rarefaction analyses, indicating the  $\alpha$ -diversity of PVC biofilm bacterial communities at the sampling period of 3, 6, 9, 12, 18, 30, 42 and 56 weeks.

## References

- AGOGUÉ, H., LAMY, D., NEAL, P. R., SOGIN, M. L. & HERNDL, G. J. 2011. Water mass-specificity of bacterial communities in the North Atlantic revealed by massively parallel sequencing. *Molecular ecology*, 20, 258-274.
- AL-OUFI, H., MCLEAN, E., KUMAR, A., CLAEREBOUDT, M. & AL-HABSI, M. 2004. The effects of solar radiation upon breaking strength and elongation of fishing nets. *Fisheries research*, 66, 115-119.
- AL-ABDULRAZZAK, S. & JABARIN, S. A. 2002. Processing characteristics of poly (ethylene terephthalate): hydrolytic and thermal degradation. *Polymer international*, 51, 164-173.
- ALEXANDER, M. 1975. Environmental and microbiological problems arising from recalcitrant molecules. *Microbial ecology*, 2, 17-27.
- ALLISON, S. D. & MARTINY, J. B. H. 2008. Resistance, resilience, and redundancy in microbial communities. *Proceedings of the National Academy of Sciences*, 105, 11512.
- ALONSO-SÁEZ, L. & GASOL, J. M. 2007. Seasonal variations in the contributions of different bacterial groups to the uptake of low-molecular-weight compounds in northwestern Mediterranean coastal waters. *Applied and environmental microbiology*, 73, 3528-3535.
- ALOTHMAN, O., SALAHUDEEN, S., ELLEITHY, R. & AL-ZAHRANI, S. M. 2012. Formation of Vinylidene in Polypropylene/Ethylene Vinyl Acetate (PP/EVA) Blends During Degradation. *Polymer-Plastics Technology and Engineering*, 51, 540-547.
- ALVAREZ-ZEFERINO, J. C., BELTRÁN-VILLAVICENCIO, M. & VÁZQUEZ-MORILLAS, A. 2015. Degradation of plastics in seawater in laboratory. *Open Journal of Polymer Chemistry*, 5, 55.
- AMARAL-ZETTLER, L., ARTIGAS, L. F., BAROSS, J., BHARATHI, L., BOETIUS, A., CHANDRAMOHAN, D., HERNDL, G., KOGURE, K., NEAL, P. & PEDRÓS-ALIÓ, C. 2010. A global census of marine microbes. *Life in the world's Oceans: Diversity, Distribution and Abundance*, 223-245.
- AMARAL-ZETTLER, L. A., ZETTLER, E. R., SLIKAS, B., BOYD, G. D., MELVIN, D. W., MORRALL, C. E., PROSKUROWSKI, G. & MINCER, T. J. 2015. The biogeography of the Plastisphere: implications for policy. *Frontiers in Ecology and the Environment*, 13, 541-546.
- ANDERSON, M., GORLEY, R. & CLARKE, R. 2008. PERMANOVA+ for PRIMER: guide to software and statistical methods. PRIMER-E Ltd, Plymouth, UK.
- ANDERSON, M. J. 2006. Distance-based tests for homogeneity of multivariate dispersions. *Biometrics*, 62, 245-253.
- ANDERSON, M. J. & WALSH, D. C. 2013. PERMANOVA, ANOSIM, and the Mantel test in the face of heterogeneous dispersions: what null hypothesis are you testing? *Ecological monographs*, 83, 557-574.

- ANDRADY, A., PEGRAM, J. & TROPSHA, Y. 1993a. Changes in carbonyl index and average molecular weight on embrittlement of enhanced-photodegradable polyethylenes. *Journal of environmental polymer degradation*, 1, 171-179.
- ANDRADY, A. L. 1990. Weathering of polyethylene (LDPE) and enhanced photodegradable polyethylene in the marine environment. *Journal of Applied Polymer Science*, 39, 363-370.
- ANDRADY, A. L. 1994. Assessment of Environmental Biodegradation of Synthetic Polymers. *Journal of Macromolecular Science, Part C*, 34, 25-76.
- ANDRADY, A. L. 2011. Microplastics in the marine environment. *Marine Pollution Bulletin*, 62, 1596-1605.
- ANDRADY, A. L. 2015a. Degradation of Plastics in the Environment. *Plastics and Environmental Sustainability*. John Wiley & Sons, Inc, Hoboken, NJ. doi: 10.1002/9781119009405.ch6.
- ANDRADY, A. L. 2015b. Plastics in the Oceans. *Plastics and Environmental Sustainability*. John Wiley & Sons, Inc, Hoboken, NJ.
- ANDRADY, A. L. 2017. The plastic in microplastics: A review. *Marine Pollution Bulletin*, 119, 12-22.
- ANDRADY, A. L. & NEAL, M. A. 2009. Applications and societal benefits of plastics. *Phil Trans R Soc B*, 364.
- ANDRADY, A. L., PEGRAM, J. E. & SONG, Y. 1993b. Studies on enhanced degradable plastics. II. Weathering of enhanced photodegradable polyethylenes under marine and freshwater floating exposure. *Journal of environmental polymer degradation*, 1, 117-126.
- ARRIGO, K. R. 2004. Marine microorganisms and global nutrient cycles. *Nature*, 437, 349.
- ARTHAM, T., SUDHAKAR, M., VENKATESAN, R., MADHAVAN NAIR, C., MURTY, K. V. G. K. & DOBLE, M. 2009a. Biofouling and stability of synthetic polymers in sea water. *International Biodeterioration & Biodegradation*, 63, 884-890.
- ARTHAM, T., SUDHAKAR, M., VENKATESAN, R., NAIR, C. M., MURTY, K. & DOBLE, M. 2009b. Biofouling and stability of synthetic polymers in sea water. *International Biodeterioration & Biodegradation*, 63, 884-890.
- ASENSIO, R. C., MOYA, M. S. A., DE LA ROJA, J. M. & GÓMEZ, M. 2009. Analytical characterization of polymers used in conservation and restoration by ATR-FTIR spectroscopy. *Analytical and Bioanalytical Chemistry*, 395, 2081-2096.
- ASTM-INTERNATIONAL 2009. *ASTM D6691-09 Standard test method for determining aerobic biodegradation of plastic materials in the marine environment by a defined microbial consortium or natural sea water inoculum*, pp. 1–4. West Conshohocken, PA: ASTM International, ASTM-International.
- ASTM-INTERNATIONAL 2012. *ASTM D882-12 Standard Test Method for Tensile Properties of Thin Plastic Sheeting*, pp. 1–10. West Conshohocken, PA: ASTM International, ASTM-International

- AUTA, H. S., EMENIKE, C. U. & FAUZIAH, S. H. 2017. Screening of *Bacillus* strains isolated from mangrove ecosystems in Peninsular Malaysia for microplastic degradation. *Environmental Pollution*, 231, 1552-1559.
- AUTA, H. S., EMENIKE, C. U., JAYANTHI, B. & FAUZIAH, S. H. 2018. Growth kinetics and biodeterioration of polypropylene microplastics by *Bacillus* sp. and *Rhodococcus* sp. isolated from mangrove sediment. *Marine Pollution Bulletin*, 127, 15-21.
- AVIO, C. G., GORBI, S., MILAN, M., BENEDETTI, M., FATTORINI, D., D'ERRICO, G., PAULETTO, M., BARGELLONI, L. & REGOLI, F. 2015. Pollutants bioavailability and toxicological risk from microplastics to marine mussels. *Environmental Pollution*, 198, 211-222.
- AVIO, C. G., GORBI, S. & REGOLI, F. 2017. Plastics and microplastics in the oceans: From emerging pollutants to emerged threat. *Marine Environmental Research*, 128, 2-11.
- AZAM, F., FENCHEL, T., FIELD, J. G., GRAY, J., MEYER-REIL, L. & THINGSTAD, F. 1983. The ecological role of water-column microbes in the sea. *Marine ecology progress series*, 257-263.
- AZEVEDO, H. S. & REIS, R. L. 2005. Understanding the enzymatic degradation of biodegradable polymers and strategies to control their degradation rate. *Biodegradable systems in tissue engineering and regenerative medicine*. CRC Press, Boca Raton, pp 177-201.
- AZZARELLO, M. Y. & VAN VLEET, E. S. 1987. Marine birds and plastic pollution. *Marine Ecology Progress Series*, 37, 295-303.
- BAEKELAND, L. H. 1909. The Synthesis, Constitution, and Uses of Bakelite. *Industrial & Engineering Chemistry*, 1, 149-161.
- BALASUBRAMANIAN, V., NATARAJAN, K., HEMAMBIKA, B., RAMESH, N., SUMATHI, C., KOTTAIMUTHU, R. & RAJESH KANNAN, V. 2010. High-density polyethylene (HDPE)-degrading potential bacteria from marine ecosystem of Gulf of Mannar, India. *Letters in applied microbiology*, 51, 205-211.
- BARNES, D. K., GALGANI, F., THOMPSON, R. C. & BARLAZ, M. 2009. Accumulation and fragmentation of plastic debris in global environments. *Philosophical Transactions of the Royal Society of London B: Biological Sciences*, 364, 1985-1998.
- BEAUMONT, N. J., AANESEN, M., AUSTEN, M. C., BÖRGER, T., CLARK, J. R., COLE, M., HOOPER, T., LINDEQUE, P. K., PASCOE, C. & WYLES, K. J. 2019. Global ecological, social and economic impacts of marine plastic. *Marine Pollution Bulletin*, 142, 189-195.
- BECK, C. A. & BARROS, N. B. 1991. The impact of debris on the Florida manatee. *Marine Pollution Bulletin*, 22, 508-510.
- BEJGARN, S., MACLEOD, M., BOGDAL, C. & BREITHOLTZ, M. 2015. Toxicity of leachate from weathering plastics: An exploratory screening study with *Nitocra spinipes*. *Chemosphere*, 132, 114-119.

- BELTRAN, M. & MARCILLA, A. 1997. Fourier transform infrared spectroscopy applied to the study of PVC decomposition. *European polymer journal*, 33, 1135-1142.
- BESSELING, E., WEGNER, A., FOEKEMA, E. M., VAN DEN HEUVEL-GREVE, M. J. & KOELMANS, A. A. 2013. Effects of Microplastic on Fitness and PCB Bioaccumulation by the Lugworm *Arenicola marina* (L.). *Environmental Science & Technology*, 47, 593-600.
- BETTAS ARDISSON, G., TOSIN, M., BARBALE, M. & DEGLI-INNOCENTI, F. 2014. Biodegradation of plastics in soil and effects on nitrification activity. A laboratory approach. *Frontiers in microbiology*, 5, 710-710.
- BEYLER, C. L. & HIRSCHLER, M. M. 2002. Thermal decomposition of polymers. *SFPE handbook of fire protection engineering*, 2, 110-131.
- BIBER, N. F. A., FOGGO, A. & THOMPSON, R. C. 2019. Characterising the deterioration of different plastics in air and seawater. *Marine Pollution Bulletin*, 141, 595-602.
- BIŽIĆ-IONESCU, M., IONESCU, D. & GROSSART, H.-P. 2018. Organic Particles: Heterogeneous Hubs for Microbial Interactions in Aquatic Ecosystems. *Frontiers in Microbiology*, 9, 2569.
- BIŽIĆ-IONESCU, M., ZEDER, M., IONESCU, D., ORLIĆ, S., FUCHS, B. M., GROSSART, H. P. & AMANN, R. 2015. Comparison of bacterial communities on limnic versus coastal marine particles reveals profound differences in colonization. *Environmental microbiology*, 17, 3500-3514.
- BLANCO, L. P., EVANS, M. L., SMITH, D. R., BADTKE, M. P. & CHAPMAN, M. R. 2012. Diversity, biogenesis and function of microbial amyloids. *Trends in microbiology*, 20, 66-73.
- BOKRIA, J. G. & SCHLICK, S. 2002. Spatial effects in the photodegradation of poly(acrylonitrile–butadiene–styrene): a study by ATR-FTIR. *Polymer*, 43, 3239-3246.
- BOLLINGER, A., THIES, S., KNIIPS-GRÜNHAGEN, E., GERTZEN, C., KOBUS, S., HÖPPNER, A., FERRER, M., GOHLKE, H., SMITS, S. H. & JAEGER, K.-E. 2020. A novel polyester hydrolase from the marine bacterium *Pseudomonas aestusnigri*—Structural and functional insights. *Frontiers in microbiology*, 11, 114.
- BOND, T., FERRANDIZ-MAS, V., FELIPE-SOTELO, M. & VAN SEBILLE, E. 2018. The occurrence and degradation of aquatic plastic litter based on polymer physicochemical properties: A review. *Critical Reviews in Environmental Science and Technology*, 48, 685-722.
- BOOTH, G., COOPER, A. & ROBB, J. 1968. Bacterial degradation of plasticized PVC. *Journal of Applied Bacteriology*, 31, 305-310.
- BOWMAN, J. P., MCCAMMON, S. A., BROWN, M. V., NICHOLS, D. S. & MCMEEKIN, T. A. 1997. Diversity and association of psychrophilic bacteria in Antarctic sea ice. *Applied and Environmental Microbiology*, 63, 3068.

- BOZAL, N., MONTES, M. J. & MERCADÉ, E. 2007. *Pseudomonas guineae* sp. nov., a novel psychrotolerant bacterium from an Antarctic environment. *International Journal of Systematic and Evolutionary Microbiology*, 57, 2609-2612.
- BRANDON, J., GOLDSTEIN, M. & OHMAN, M. D. 2016. Long-term aging and degradation of microplastic particles: Comparing in situ oceanic and experimental weathering patterns. *Marine Pollution Bulletin*, 110, 299-308.
- BRIASSOULIS, D., DEJEAN, C. & PICUNO, P. 2010. Critical Review of Norms and Standards for Biodegradable Agricultural Plastics Part II: Composting. *Journal of Polymers and the Environment*, 18, 364-383.
- BROUWER, R., HADZHIYSKA, D., IOAKEIMIDIS, C. & OUDERDORP, H. 2017. The social costs of marine litter along European coasts. *Ocean & coastal management*, 138, 38-49.
- BROWNE, M. A., CRUMP, P., NIVEN, S. J., TEUTEN, E., TONKIN, A., GALLOWAY, T. & THOMPSON, R. 2011. Accumulation of microplastic on shorelines worldwide: sources and sinks. *Environmental science & technology*, 45, 9175-9179.
- BROWNE, M. A., GALLOWAY, T. & THOMPSON, R. 2007. Microplastic—an emerging contaminant of potential concern? *Integrated Environmental Assessment and Management*, 3, 559-561.
- BROWNE, M. A., GALLOWAY, T. S. & THOMPSON, R. C. 2010. Spatial Patterns of Plastic Debris along Estuarine Shorelines. *Environmental Science & Technology*, 44, 3404-3409.
- BROWNE, M. A., NIVEN, S. J., GALLOWAY, T. S., ROWLAND, S. J. & THOMPSON, R. C. 2013. Microplastic moves pollutants and additives to worms, reducing functions linked to health and biodiversity. *Current Biology*, 23, 2388-2392.
- BRYANT, J. A., CLEMENTE, T. M., VIVIANI, D. A., FONG, A. A., THOMAS, K. A., KEMP, P., KARL, D. M., WHITE, A. E. & DELONG, E. F. 2016. Diversity and activity of communities inhabiting plastic debris in the North Pacific Gyre. *MSystems*, 1, e00024-16.
- BUNSE, C. & PINHASSI, J. 2017. Marine Bacterioplankton Seasonal Succession Dynamics. *Trends in Microbiology*, 25, 494-505.
- BUSSCHER, H. J., NORDE, W., SHARMA, P. K. & VAN DER MEI, H. C. 2010. Interfacial re-arrangement in initial microbial adhesion to surfaces. *Current Opinion in Colloid & Interface Science*, 15, 510-517.
- CAI, L., WANG, J., PENG, J., WU, Z. & TAN, X. 2018. Observation of the degradation of three types of plastic pellets exposed to UV irradiation in three different environments. *Science of The Total Environment*, 628-629, 740-747.
- CALLOW, M. E. & FLETCHER, R. L. 1994. The influence of low surface energy materials on bioadhesion — a review. *International Biodeterioration & Biodegradation*, 34, 333-348.
- CAMPAGNA, C., FALABELLA, V. & LEWIS, M. 2007. Entanglement of southern elephant seals in squid fishing gear. *Marine Mammal Science*, 23, 414-418.

- CAPORASO, J. G., LAUBER, C. L., WALTERS, W. A., BERG-LYONS, D., LOZUPONE, C. A., TURNBAUGH, P. J., FIERER, N. & KNIGHT, R. 2011. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. *Proceedings of the national academy of sciences*, 108, 4516-4522.
- CARPENTER, E. J., ANDERSON, S. J., HARVEY, G. R., MIKLAS, H. P. & PECK, B. B. 1972. Polystyrene spherules in coastal waters. *Science*, 178, 749-750.
- CARPENTER, E. J. & SMITH, K. L. 1972. Plastics on the Sargasso Sea Surface. *Science*, 175, 1240-1241.
- CARR, S. A., LIU, J. & TESORO, A. G. 2016. Transport and fate of microplastic particles in wastewater treatment plants. *Water research*, 91, 174-182.
- CARSON, H. S., NERHEIM, M. S., CARROLL, K. A. & ERIKSEN, M. 2013. The plastic-associated microorganisms of the North Pacific Gyre. *Mar Pollut Bull*, 75, 126-132.
- CARVALHO, M. F., FERREIRA, M. I. M., MOREIRA, I. S., CASTRO, P. M. & JANSSEN, D. B. 2006. Degradation of fluorobenzene by Rhizobiales strain F11 via ortho cleavage of 4-fluorocatechol and catechol. *Applied and environmental microbiology*, 72, 7413-7417.
- CAVICCHIOLI, R., OSTROWSKI, M., FEGATELLA, F., GOODCHILD, A. & GUIXA-BOIXEREU, N. 2003. Life under nutrient limitation in oligotrophic marine environments: an eco/physiological perspective of *Sphingopyxis alaskensis* (formerly *Sphingomonas alaskensis*). *Microbial ecology*, 45, 203-217.
- CEDERVALL, T., HANSSON, L.-A., LARD, M., FROHM, B. & LINSE, S. 2012. Food Chain Transport of Nanoparticles Affects Behaviour and Fat Metabolism in Fish. *PLOS ONE*, 7, e32254.
- CHAMAS, A., MOON, H., ZHENG, J., QIU, Y., TABASSUM, T., JANG, J. H., ABU-OMAR, M., SCOTT, S. L. & SUH, S. 2020. Degradation Rates of Plastics in the Environment. *ACS Sustainable Chemistry & Engineering*, 8, 3494-3511.
- CHEN, C.-L., MAKI, J. S., RITTSCHOF, D. & TEO, S. L.-M. 2013. Early marine bacterial biofilm on a copper-based antifouling paint. *International Biodeterioration & Biodegradation*, 83, 71-76.
- CHI, W.-J., PARK, J.-S., KANG, D.-K. & HONG, S.-K. 2014. Production and Characterization of a Novel Thermostable Extracellular Agarase from *Pseudoalteromonas hodoensis* Newly Isolated from the West Sea of South Korea. *Applied Biochemistry and Biotechnology*, 173, 1703-1716.
- CHIELLINI, E., CORTI, A. & SOLARO, R. 1999. Biodegradation of poly(vinyl alcohol) based blown films under different environmental conditions11Part of work herewith reported was presented at the 5th Scientific Workshop on Biodegradable Polymers and Plastics, Stockholm (Se) June 1998. *Polymer Degradation and Stability*, 64, 305-312.
- CHIELLINI, E., CORTI, A. & SWIFT, G. 2003. Biodegradation of thermally-oxidized, fragmented low-density polyethylenes. *Polymer degradation and stability*, 81, 341-351.



- CHIU, J., ZHANG, R., WANG, H., THIYAGARAJAN, V. & QIAN, P. 2008. Nutrient effects on intertidal community: from bacteria to invertebrates. *Marine Ecology Progress Series*, 358, 41-50.
- CHUBARENKO, I., BAGAEV, A., ZOBKOV, M. & ESIUKOVA, E. 2016. On some physical and dynamical properties of microplastic particles in marine environment. *Marine Pollution Bulletin*, 108, 105-112.
- CLARKE, K. & GORLEY, R. 2015. PRIMER v7: User manual/tutorial (p. 300). *Plymouth, UK: PRIMER-E Ltd.*
- CLARKE, K. R. 1993. Non-parametric multivariate analyses of changes in community structure. *Aust J Ecol*, 18.
- CLARKE, K. R., SOMERFIELD, P. J. & CHAPMAN, M. G. 2006. On resemblance measures for ecological studies, including taxonomic dissimilarities and a zero-adjusted Bray–Curtis coefficient for denuded assemblages. *Journal of Experimental Marine Biology and Ecology*, 330, 55-80.
- COATES, J. 2006. Interpretation of Infrared Spectra, A Practical Approach. In: MCKELVY, R. A. M. A. M. L. (ed.) *Encyclopedia of Analytical Chemistry: applications, theory and instrumentation.*: doi:10.1002/9780470027318.a5606.
- COLE, M., LINDEQUE, P., HALSBAND, C. & GALLOWAY, T. S. 2011. Microplastics as contaminants in the marine environment: A review. *Mar Poll Bull*, 62, 2588-2597.
- COMTE, J. & DEL GIORGIO, P. A. 2011. Composition Influences the Pathway but not the Outcome of the Metabolic Response of Bacterioplankton to Resource Shifts. *PLOS ONE*, 6, e25266.
- COOKSEY, K. E. & WIGGLESWORTH-COOKSEY, B. 1995. Adhesion of bacteria and diatoms to surfaces in the sea: a review. *Aquatic Microbial Ecology*, 09, 87-96.
- COOPER, D. A. 2012. *Effects of Chemical and Mechanical Weathering Processes on the Degradation of Plastic Debris on Marine Beaches*. (Doctoral dissertation, The University of Western Ontario).
- COOPER, D. A. & CORCORAN, P. L. 2010. Effects of mechanical and chemical processes on the degradation of plastic beach debris on the island of Kauai, Hawaii. *Marine Pollution Bulletin*, 60, 650-654.
- CORCORAN, P. L., BIESINGER, M. C. & GRIFI, M. 2009. Plastics and beaches: A degrading relationship. *Marine Pollution Bulletin*, 58, 80-84.
- CÓZAR, A., ECHEVARRÍA, F., GONZÁLEZ-GORDILLO, J. I., IRIGOIEN, X., ÚBEDA, B., HERNÁNDEZ-LEÓN, S., PALMA, Á. T., NAVARRO, S., GARCÍA-DE-LOMAS, J. & RUIZ, A. 2014. Plastic debris in the open ocean. *Proceedings of the National Academy of Sciences*, 111, 10239-10244.
- DA COSTA, J. P., NUNES, A. R., SANTOS, P. S. M., GIRÃO, A. V., DUARTE, A. C. & ROCHA-SANTOS, T. 2018. Degradation of polyethylene microplastics in seawater: Insights into the environmental degradation of polymers. *Journal of Environmental Science and Health, Part A*, 53, 866-875.

- DANG, H., KLOTZ, M. G., LOVELL, C. R. & SIEVERT, S. M. 2019. Editorial: The Responses of Marine Microorganisms, Communities and Ecofunctions to Environmental Gradients. *Frontiers in Microbiology*, 10, 115.
- DANG, H., LI, T., CHEN, M. & HUANG, G. 2008. Cross-ocean distribution of Rhodobacterales bacteria as primary surface colonizers in temperate coastal marine waters. *Appl Environ Microbiol*, 74, 52-60.
- DANG, H. & LOVELL, C. R. 2000. Bacterial primary colonization and early succession on surfaces in marine waters as determined by amplified rRNA gene restriction analysis and sequence analysis of 16S rRNA genes. *Appl Environ Microbiol*, 66, 467-475.
- DANG, H. & LOVELL, C. R. 2016. Microbial surface colonization and biofilm development in marine environments. *Microbiol. Mol. Biol. Rev.*, 80, 91-138.
- DANKO, A. S., LUO, M., BAGWELL, C. E., BRIGMON, R. L. & FREEDMAN, D. L. 2004. Involvement of Linear Plasmids in Aerobic Biodegradation of Vinyl Chloride. *Applied and Environmental Microbiology*, 70, 6092-6097.
- DANKO, A. S., SASKI, C. A., TOMKINS, J. P. & FREEDMAN, D. L. 2006. Involvement of coenzyme M during aerobic biodegradation of vinyl chloride and ethene by *Pseudomonas putida* strain AJ and *Ochrobactrum* sp. strain TD. *Appl. Environ. Microbiol.*, 72, 3756-3758.
- DAS, G., BORDOLOI, N. K., RAI, S. K., MUKHERJEE, A. K. & KARAK, N. 2012. Biodegradable and biocompatible epoxidized vegetable oil modified thermostable poly (vinyl chloride): Thermal and performance characteristics post biodegradation with *Pseudomonas aeruginosa* and *Achromobacter* sp. *Journal of hazardous materials*, 209, 434-442.
- DAS, K. & MUKHERJEE, A. K. 2007. Crude petroleum-oil biodegradation efficiency of *Bacillus subtilis* and *Pseudomonas aeruginosa* strains isolated from a petroleum-oil contaminated soil from North-East India. *Bioresource Technology*, 98, 1339-1345.
- DAS, M. P. & KUMAR, S. 2015. An approach to low-density polyethylene biodegradation by *Bacillus amyloliquefaciens*. *3 Biotech*, 5, 81-86.
- DAU, B. K., GILARDI, K. V. K., GULLAND, F. M., HIGGINS, A., HOLCOMB, J. B., LEGER, J. S. & ZICCARDI, M. H. 2009. Fishing gear-related injury in California marine wildlife. *Journal of Wildlife Diseases*, 45, 355-362.
- DAVEY, M. E. & O'TOOLE, G. A. 2000. Microbial biofilms: from ecology to molecular genetics. *Microbiology and molecular biology reviews*, 64, 847-867.
- DAVID, I. K., NEILAN, M. K., MYA, B., NANCY, K. & FOREST, R. 2006. Role of elevated organic carbon levels and microbial activity in coral mortality. *Marine Ecology Progress Series*, 314, 119-125.
- DE AZEVEDO E SILVA, C. E., AZEREDO, A., LAILSON-BRITO, J., TORRES, J. P. M. & MALM, O. 2007. Polychlorinated biphenyls and DDT in swordfish (*Xiphias gladius*) and blue shark (*Prionace glauca*) from Brazilian coast. *Chemosphere*, 67, S48-S53.

- DE STEPHANIS, R., GIMÉNEZ, J., CARPINELLI, E., GUTIERREZ-EXPOSITO, C. & CAÑADAS, A. 2013. As main meal for sperm whales: Plastics debris. *Marine Pollution Bulletin*, 69, 206-214.
- DE TENDER, C., DEVRIESE, L. I., HAEGEMAN, A., MAES, S., VANGEYTE, J., CATTRIJSSE, A., DAWYNDT, P. & RUTTINK, T. 2017a. Temporal Dynamics of Bacterial and Fungal Colonization on Plastic Debris in the North Sea. *Environmental Science & Technology*, 51, 7350-7360.
- DE TENDER, C., SCHLUNDT, C., DEVRIESE, L., MINCER, T., ZETTLER, E. & AMARAL-ZETTLER, L. 2017b. A review of microscopy and comparative molecular-based methods to characterize “Plastisphere” communities. *Analytical Methods*, 9, 2132-2143.
- DE TENDER, C. A., DEVRIESE, L. I., HAEGEMAN, A., MAES, S., RUTTINK, T. & DAWYNDT, P. 2015. Bacterial Community Profiling of Plastic Litter in the Belgian Part of the North Sea. *Environmental Science & Technology*, 49, 9629-9638.
- DEBROAS, D., MONE, A. & TER HALLE, A. 2017. Plastics in the North Atlantic garbage patch: A boat-microbe for hitchhikers and plastic degraders. *Science of The Total Environment*, 599-600, 1222-1232.
- DEL GIORGIO, P. A. & DUARTE, C. M. 2002. Respiration in the open ocean. *Nature*, 420, 379.
- DEPLEDGE, M., GALGANI, F., PANTI, C., CALIANI, I., CASINI, S. & FOSSI, M. 2013. Plastic litter in the sea. *Marine environmental research*, 92, 279-281.
- DERRAIK, J. G. B. 2002. The pollution of the marine environment by plastic debris: a review. *Mar Pollut Bull*, 44, 842-852.
- DEVI, R. S., KANNAN, V. R., NIVAS, D., KANNAN, K., CHANDRU, S. & ANTONY, A. R. 2015. Biodegradation of HDPE by *Aspergillus* spp. from marine ecosystem of Gulf of Mannar, India. *Marine Pollution Bulletin*, 96, 32-40.
- DONELLI, I., TADDEI, P., SMET, P. F., POELMAN, D., NIERSTRASZ, V. A. & FREDDI, G. 2009. Enzymatic surface modification and functionalization of PET: a water contact angle, FTIR, and fluorescence spectroscopy study. *Biotechnology and bioengineering*, 103, 845-856.
- DONEY, S. C., FABRY, V. J., FEELY, R. A. & KLEYPAS, J. A. 2009. Ocean Acidification: The Other CO<sub>2</sub> Problem. *Annual Review of Marine Science*, 1, 169-192.
- DONLAN, R. M. 2002. Biofilms: microbial life on surfaces. *Emerging infectious diseases*, 8, 881.
- DUHEC, A. V., JEANNE, R. F., MAXIMENKO, N. & HAFNER, J. 2015. Composition and potential origin of marine debris stranded in the Western Indian Ocean on remote Alphonse Island, Seychelles. *Marine pollution bulletin*, 96, 76-86.
- DUSSUD, C. & GHIGLIONE, J.-F. 2014. Bacterial degradation of synthetic plastics. *CIESM Workshop Monograph*, 46, 49-54.

- DUSSUD, C., HUDEC, C., GEORGE, M., FABRE, P., HIGGS, P., BRUZAUD, S., DELORT, A.-M., EYHERAGUIBEL, B., MEISTERTZHEIM, A.-L., JACQUIN, J., CHENG, J., CALLAC, N., ODOBEL, C., RABOUILLE, S. & GHIGLIONE, J.-F. 2018. Colonization of Non-biodegradable and Biodegradable Plastics by Marine Microorganisms. *Frontiers in Microbiology*, 9, 1571.
- DUTTA, C. & PAUL, S. 2012. Microbial lifestyle and genome signatures. *Current genomics*, 13, 153-162.
- DUWEZ, A. S. & NYSTEN, B. 2001. Mapping Aging Effects on Polymer Surfaces: Specific Detection of Additives by Chemical Force Microscopy. *Langmuir*, 17, 8287-8292.
- DWIVEDI, C., PANDEY, I., PANDEY, H., RAMTEKE, P. W., PANDEY, A. C., MISHRA, S. B. & PATIL, S. 2017. Chapter 9 - Electrospun Nanofibrous Scaffold as a Potential Carrier of Antimicrobial Therapeutics for Diabetic Wound Healing and Tissue Regeneration. In: GRUMEZESCU, A. M. (ed.) *Nano- and Microscale Drug Delivery Systems*. Elsevier.
- EGAN, S., HARDER, T., BURKE, C., STEINBERG, P., KJELLEBERG, S. & THOMAS, T. 2013. The seaweed holobiont: understanding seaweed–bacteria interactions. *FEMS Microbiology Reviews*, 37, 462-476.
- EICH, A., MILDENBERGER, T., LAFORSCH, C. & WEBER, M. 2015. Biofilm and Diatom Succession on Polyethylene (PE) and Biodegradable Plastic Bags in Two Marine Habitats: Early Signs of Degradation in the Pelagic and Benthic Zone? *PLOS ONE*, 10, e0137201.
- ELIFANTZ, H., HORN, G., AYON, M., COHEN, Y. & MINZ, D. 2013. Rhodobacteraceae are the key members of the microbial community of the initial biofilm formed in Eastern Mediterranean coastal seawater. *FEMS Microbiology Ecology*, 85, 348-357.
- ELSAYED, Y., REFAAT, J., ABDELMOHSEN, U. R. & FOUAD, M. A. 2017. The Genus *Rhodococcus* as a source of novel bioactive substances: A review. *J. Pharma. Phytochem*, 6, 83-92.
- ERIKSEN, M., MAXIMENKO, N., THIEL, M., CUMMINS, A., LATTIN, G., WILSON, S., HAFNER, J., ZELLERS, A. & RIFMAN, S. 2013. Plastic pollution in the South Pacific subtropical gyre. *Marine pollution bulletin*, 68, 71-76.
- EUROPEAN BIOPLASTICS 2018. Bioplastics market data 2018 *Global production capacities of bioplastics 2018-2023*, [https://www.european-bioplastics.org/wp-content/uploads/2016/02/Report\\_Bioplastics-Market-Data\\_2018.pdf](https://www.european-bioplastics.org/wp-content/uploads/2016/02/Report_Bioplastics-Market-Data_2018.pdf).
- FALKOWSKI, P. G., FENCHEL, T. & DELONG, E. F. 2008. The Microbial Engines That Drive Earth's Biogeochemical Cycles. *Science*, 320, 1034.
- FAROON, O. & RUIZ, P. 2016. Polychlorinated biphenyls: New evidence from the last decade. *Toxicology and industrial health*, 32, 1825-1847.
- FAZEY, F. M. C. & RYAN, P. G. 2016. Biofouling on buoyant marine plastics: An experimental study into the effect of size on surface longevity. *Environmental Pollution*, 210, 354-360.

- FLEMMING, H.-C. 1998. Relevance of biofilms for the biodeterioration of surfaces of polymeric materials. *Polymer Degradation and Stability*, 59, 309-315.
- FLEMMING, H.-C. & WINGENDER, J. 2010. The biofilm matrix. *Nature Reviews Microbiology*, 8, 623-633.
- FLEMMING, H.-C., WINGENDER, J., SZEWZYK, U., STEINBERG, P., RICE, S. A. & KJELLEBERG, S. 2016. Biofilms: an emergent form of bacterial life. *Nature Reviews Microbiology*, 14, 563.
- FONTANELLA, S., BONHOMME, S., KOUTNY, M., HUSAROVA, L., BRUSSON, J.-M., COURDAVAULT, J.-P., PITTERI, S., SAMUEL, G., PICHON, G., LEMAIRE, J. & DELORT, A.-M. 2010. Comparison of the biodegradability of various polyethylene films containing pro-oxidant additives. *Polymer Degradation and Stability*, 95, 1011-1021.
- FOTOPOULOU, K. N. & KARAPANAGIOTI, H. K. 2012. Surface properties of beached plastic pellets. *Marine Environmental Research*, 81, 70-77.
- FOTOPOULOU, K. N. & KARAPANAGIOTI, H. K. 2015. Surface properties of beached plastics. *Environmental Science and Pollution Research*, 22, 11022-11032.
- FOWLER, C. W. 1987. Marine debris and northern fur seals: A case study. *Marine Pollution Bulletin*, 18, 326-335.
- FRANÇOIS-HEUDE, A., RICHAUD, E., DESNOUX, E. & COLIN, X. 2014. Influence of temperature, UV-light wavelength and intensity on polypropylene photothermal oxidation. *Polymer Degradation and Stability*, 100, 10-20.
- FRÈRE, L., MAIGNIEN, L., CHALOPIN, M., HUVET, A., RINNERT, E., MORRISON, H., KERNINON, S., CASSONE, A.-L., LAMBERT, C., REVEILLAUD, J. & PAUL-PONT, I. 2018. Microplastic bacterial communities in the Bay of Brest: Influence of polymer type and size. *Environmental Pollution*, 242, 614-625.
- FRIAS, J. P. G. L., SOBRAL, P. & FERREIRA, A. M. 2010. Organic pollutants in microplastics from two beaches of the Portuguese coast. *Marine Pollution Bulletin*, 60, 1988-1992.
- FRIED, J. R. 1995. *Polymer Science & Technology: Introduction to Polymer Science*. 3rd edn. Prentice Hall, Upper Saddle River, 4-9.
- FUHRMAN, J. A., SLEETER, T. D., CARLSON, C. A. & PROCTOR, L. M. 1989. Dominance of bacterial biomass in the Sargasso Sea and its ecological implications. *Marine Ecology Progress Series*, 207-217.
- GALL, S. C. & THOMPSON, R. C. 2015. The impact of debris on marine life. *Marine Pollution Bulletin*, 92, 170-179.
- GEWERT, B., PLASSMANN, M. M. & MACLEOD, M. 2015. Pathways for degradation of plastic polymers floating in the marine environment. *Environmental Science: Processes & Impacts*, 17, 1513-1521.
- GEYER, R., JAMBECK, J. R. & LAW, K. L. 2017. Production, use, and fate of all plastics ever made. *Science advances*, 3, e1700782.

- GIACOMUCCI, L., RADDADI, N., SOCCIO, M., LOTTI, N. & FAVA, F. 2019. Polyvinyl chloride biodegradation by *Pseudomonas citronellolis* and *Bacillus flexus*. *New Biotechnology*, 52, 35-41.
- GIGAULT, J., HALLE, A. T., BAUDRIMONT, M., PASCAL, P.-Y., GAUFFRE, F., PHI, T.-L., EL HADRI, H., GRASSL, B. & REYNAUD, S. 2018. Current opinion: What is a nanoplastic? *Environmental Pollution*, 235, 1030-1034.
- GIJSMAN, P., MEIJERS, G. & VITARELLI, G. 1999. Comparison of the UV-degradation chemistry of polypropylene, polyethylene, polyamide 6 and polybutylene terephthalate. *Polymer Degradation and Stability*, 65, 433-441.
- GILARDI, K. V. K., CARLSON-BREMER, D., JUNE, J. A., ANTONELIS, K., BROADHURST, G. & COWAN, T. 2010. Marine species mortality in derelict fishing nets in Puget Sound, WA and the cost/benefits of derelict net removal. *Marine Pollution Bulletin*, 60, 376-382.
- GOLDSTEIN, M. C. & GOODWIN, D. S. 2013. Gooseneck barnacles (*Lepas* spp.) ingest microplastic debris in the North Pacific Subtropical Gyre. *PeerJ*, 1, e184.
- GOWER, J. C. 1966. Some distance properties of latent root and vector methods used in multivariate analysis. *Biometrika*, 53, 325-338.
- GRAM, L., GROSSART, H.-P., SCHLINGLOFF, A. & KIØRBOE, T. 2002. Possible quorum sensing in marine snow bacteria: production of acylated homoserine lactones by *Roseobacter* strains isolated from marine snow. *Appl. Environ. Microbiol.*, 68, 4111-4116.
- GRIFFITHS, R. I., WHITELEY, A. S., O'DONNELL, A. G. & BAILEY, M. J. 2000. Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA-and rRNA-based microbial community composition. *Applied and environmental microbiology*, 66, 5488-5491.
- GU, J.-D. 2003. Microbiological deterioration and degradation of synthetic polymeric materials: recent research advances. *International Biodeterioration & Biodegradation*, 52, 69-91.
- GULMINE, J. V., JANISSEK, P. R., HEISE, H. M. & AKCELRUD, L. 2003. Degradation profile of polyethylene after artificial accelerated weathering. *Polymer Degradation and Stability*, 79, 385-397.
- GUO, X. & WANG, J. 2019. The chemical behaviors of microplastics in marine environment: A review. *Marine Pollution Bulletin*, 142, 1-14.
- GUTIERREZ, T., RHODES, G., MISHAMANDANI, S., BERRY, D., WHITMAN, W. B., NICHOLS, P. D., SEMPLE, K. T. & AITKEN, M. D. 2014. Polycyclic aromatic hydrocarbon degradation of phytoplankton-associated *Arenibacter* spp. and description of *Arenibacter algicola* sp. nov., an aromatic hydrocarbon-degrading bacterium. *Applied and environmental microbiology*, 80, 618-628.
- HADAD, D., GERESH, S. & SIVAN, A. 2005. Biodegradation of polyethylene by the thermophilic bacterium *Brevibacillus borstelensis*. *Journal of Applied Microbiology*, 98, 1093-1100.

- HAHLADAKIS, J. N., VELIS, C. A., WEBER, R., IACOVIDOU, E. & PURNELL, P. 2018. An overview of chemical additives present in plastics: Migration, release, fate and environmental impact during their use, disposal and recycling. *Journal of Hazardous Materials*, 344, 179-199.
- HALL, T., BIOSCIENCES, I. & CARLSBAD, C. 2011. BioEdit: an important software for molecular biology. *GERF Bull Biosci*, 2, 60-61.
- HALL, T. A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic acids symposium series*, No. 41, pp. 95–98.
- HALLE, A. T., LADIRAT, L., MARTIGNAC, M., MINGOTAUD, A. F., BOYRON, O. & PEREZ, E. 2017. To what extent are microplastics from the open ocean weathered? *Environmental Pollution*, 227, 167-174.
- HAMMER, J., KRAAK, M. H. & PARSONS, J. R. 2012. Plastics in the marine environment: the dark side of a modern gift. *Reviews of environmental contamination and toxicology*. Springer.
- HAN, S. K., NEDASHKOVSKAYA, O. I., MIKHAILOV, V. V., KIM, S. B. & BAE, K. S. 2003. *Salinibacterium amurskyense* gen. nov., sp. nov., a novel genus of the family Microbacteriaceae from the marine environment. *International Journal of Systematic and Evolutionary Microbiology*, 53, 2061-2066.
- HANSELL, D. A., CARLSON, C. A., REPETA, D. J. & SCHLITZER, R. 2009. Dissolved organic matter in the ocean: A controversy stimulates new insights. *Oceanography*, 22, 202-211.
- HARRISON, J. P., BOARDMAN, C., O'CALLAGHAN, K., DELORT, A.-M. & SONG, J. 2018a. Biodegradability standards for carrier bags and plastic films in aquatic environments: a critical review. *Royal Society Open Science*, 5, 171792.
- HARRISON, J. P., HOELLEIN, T. J., SAPP, M., TAGG, A. S., JU-NAM, Y. & OJEDA, J. J. 2018b. Microplastic-Associated Biofilms: A Comparison of Freshwater and Marine Environments. In: WAGNER, M. & LAMBERT, S. (eds.) *Freshwater Microplastics : Emerging Environmental Contaminants?* Cham: Springer International Publishing.
- HARRISON, J. P., SAPP, M., SCHRATZBERGER, M. & OSBORN, A. M. 2011. Interactions between microorganisms and marine microplastics: a call for research. *Mar Technol Soc J*, 45, 12-20.
- HARRISON, J. P., SCHRATZBERGER, M., SAPP, M. & OSBORN, A. M. 2014. Rapid bacterial colonization of low-density polyethylene microplastics in coastal sediment microcosms. *BMC Microbiology*, 14, 1-15.
- HARSHVARDHAN, K. & JHA, B. 2013. Biodegradation of low-density polyethylene by marine bacteria from pelagic waters, Arabian Sea, India. *Marine Pollution Bulletin*, 77, 100-106.
- HAYAT, R., ALI, S., AMARA, U., KHALID, R. & AHMED, I. 2010. Soil beneficial bacteria and their role in plant growth promotion: a review. *Annals of Microbiology*, 60, 579-598.

- HE, Y., QIAN, Z., ZHANG, H. & LIU, X. 2004. Alkaline degradation behavior of polyesteramide fibers: surface erosion. *Colloid and Polymer Science*, 282, 972-978.
- HEDLUND, B. P. & STALEY, J. T. 2006. Isolation and characterization of Pseudoalteromonas strains with divergent polycyclic aromatic hydrocarbon catabolic properties. *Environmental Microbiology*, 8, 178-182.
- HEIMOWSKA, A., KRASOWSKA, K. & RUTKOWSKA, M. 2011. Degradability of different packaging polymeric materials in sea water. In *Proceedings of the 12th Annual General Assembly IAMU, Green ships, eco shipping, clean seas, 12–14 June 2011*, , pp. 153–163. .
- HERRERO ACERO, E., RIBITSCH, D., STEINKELLNER, G., GRUBER, K., GREIMEL, K., EITELJOERG, I., TROTSCHA, E., WEI, R., ZIMMERMANN, W., ZINN, M., CAVACO-PAULO, A., FREDDI, G., SCHWAB, H. & GUEBITZ, G. 2011. Enzymatic Surface Hydrolysis of PET: Effect of Structural Diversity on Kinetic Properties of Cutinases from Thermobifida. *Macromolecules*, 44, 4632-4640.
- HIRAI, H., TAKADA, H., OGATA, Y., YAMASHITA, R., MIZUKAWA, K., SAHA, M., KWAN, C., MOORE, C., GRAY, H., LAURSEN, D., ZETTLER, E. R., FARRINGTON, J. W., REDDY, C. M., PEACOCK, E. E. & WARD, M. W. 2011. Organic micropollutants in marine plastics debris from the open ocean and remote and urban beaches. *Marine Pollution Bulletin*, 62, 1683-1692.
- HOLMSTRÖM, C. & KJELLEBERG, S. 1999. Marine Pseudoalteromonas species are associated with higher organisms and produce biologically active extracellular agents. *FEMS Microbiology Ecology*, 30, 285-293.
- HUNG, C.-S., ZINGARELLI, S., NADEAU, L. J., BIFFINGER, J. C., DRAKE, C. A., CROUCH, A. L., BARLOW, D. E., RUSSELL, J. N. & CROOKES-GOODSON, W. J. 2016. Carbon catabolite repression and Impranil polyurethane degradation in Pseudomonas protegens strain Pf-5. *Applied and environmental microbiology*, 82, 6080-6090.
- HUTCHINS, D. A. & FU, F. 2017. Microorganisms and ocean global change. *Nature microbiology*, 2, 17058.
- HYDE, K. D., JONES, E. B. G., LEAÑO, E., POINTING, S. B., POONYTH, A. D. & VRIJMOED, L. L. P. 1998. Role of fungi in marine ecosystems. *Biodiversity & Conservation*, 7, 1147-1161.
- IOAKEIMIDIS, C., FOTOPOULOU, K. N., KARAPANAGIOTI, H. K., GERAGA, M., ZERI, C., PAPATHANASSIOU, E., GALGANI, F. & PAPATHEODOROU, G. 2016. The degradation potential of PET bottles in the marine environment: An ATR-FTIR based approach. *Scientific Reports*, 6, 23501.
- JACKSON, G. D., BUXTON, N. G. & GEORGE, M. J. 2000. Diet of the southern opah Lampris immaculatus on the Patagonian Shelf; the significance of the squid Moroteuthis ingens and anthropogenic plastic. *Marine Ecology Progress Series*, 206, 261-271.
- JACQUIN, J., CHENG, J., ODOBEL, C., PANDIN, C., CONAN, P., PUJO-PAY, M., BARBE, V., MEISTERTZHEIM, A.-L. & GHIGLIONE, J.-F. 2019. Microbial



Ecotoxicology of Marine Plastic Debris: A Review on Colonization and Biodegradation by the “Plastisphere”. *Frontiers in Microbiology*, 10, 865.

- JAMBECK, J. R., GEYER, R., WILCOX, C., SIEGLER, T. R., PERRYMAN, M., ANDRADY, A., NARAYAN, R. & LAW, K. L. 2015. Plastic waste inputs from land into the ocean. *Science*, 347, 768-771.
- JANG, Y. C., HONG, S., LEE, J., LEE, M. J. & SHIM, W. J. 2014. Estimation of lost tourism revenue in Geoje Island from the 2011 marine debris pollution event in South Korea. *Marine Pollution Bulletin*, 81, 49-54.
- JENKINS, M. & MILLS, N. 2005. *Plastics: microstructure and engineering applications*, Elsevier.
- JENNIFER, C. & JAMES, E. B. 2004. Bacterial utilization of transient plankton-derived dissolved organic carbon and nitrogen inputs in surface ocean waters. *Aquatic Microbial Ecology*, 35, 229-241.
- JEON, H. J. & KIM, M. N. 2013. Isolation of a thermophilic bacterium capable of low-molecular-weight polyethylene degradation. *Biodegradation*, 24, 89-98.
- JIANG, P., ZHAO, S., ZHU, L. & LI, D. 2018. Microplastic-associated bacterial assemblages in the intertidal zone of the Yangtze Estuary. *Science of the total environment*, 624, 48-54.
- JOHANSSON, O. E., SMITH, D. S., SADAUSKAS-HENRIQUE, H., CIMPRICH, G., WOOD, C. M. & VAL, A. L. 2017. Photo-oxidation processes, properties of DOC, reactive oxygen species (ROS), and their potential impacts on native biota and carbon cycling in the Rio Negro (Amazonia, Brazil). *Hydrobiologia*, 789, 7-29.
- JOHNSON, M., ZARETSKAYA, I., RAYTSELIS, Y., MEREZHUK, Y., MCGINNIS, S. & MADDEN, T. L. 2008. NCBI BLAST: a better web interface. *Nucleic Acids Research*, 36, W5-W9.
- JURETSCHKO, S., TIMMERMANN, G., SCHMID, M., SCHLEIFER, K.-H., POMMERENING-RÖSER, A., KOOPS, H.-P. & WAGNER, M. 1998. Combined molecular and conventional analyses of nitrifying bacterium diversity in activated sludge: *Nitrosococcus mobilis* and *Nitrospira*-like bacteria as dominant populations. *Appl. Environ. Microbiol.*, 64, 3042-3051.
- KARAPANAGIOTI, H. & KLONTZA, I. 2007. Investigating the properties of plastic resin pellets found in the coastal areas of Lesbos Island. *Global nest. The international journal*, 9, 71-76.
- KARATAN, E. & WATNICK, P. 2009. Signals, Regulatory Networks, and Materials That Build and Break Bacterial Biofilms. *Microbiology and Molecular Biology Reviews*, 73, 310.
- KASUYA, K.-I., MITOMO, H., NAKAHARA, M., AKIBA, A., KUDO, T. & DOI, Y. 2000. Identification of a marine benthic P (3HB)-degrading bacterium isolate and characterization of its P (3HB) depolymerase. *Biomacromolecules*, 1, 194-201.
- KATHIRESAN, K. 2003. Polythene and plastics-degrading microbes from the mangrove soil. *Revista de biologia tropical*, 51, 629-633.

- KAWAI, F. & HU, X. 2009. Biochemistry of microbial polyvinyl alcohol degradation. *Applied Microbiology and Biotechnology*, 84, 227.
- KAWAI, F., KAWABATA, T. & ODA, M. 2019. Current knowledge on enzymatic PET degradation and its possible application to waste stream management and other fields. *Applied microbiology and biotechnology*, 103, 4253-4268.
- KETTNER, M. T., OBERBECKMANN, S., LABRENZ, M. & GROSSART, H.-P. 2019. The Eukaryotic Life on Microplastics in Brackish Ecosystems. *Frontiers in Microbiology*, 10, 538.
- KIJCHAVENGKUL, T., AURAS, R., RUBINO, M., NGOUAJIO, M. & THOMAS FERNANDEZ, R. 2006. Development of an automatic laboratory-scale respirometric system to measure polymer biodegradability. *Polymer Testing*, 25, 1006-1016.
- KINT, D. & MUÑOZ-GUERRA, S. 1999. A review on the potential biodegradability of poly(ethylene terephthalate). *Polymer International*, 48, 346-352.
- KIRBAŞ, Z., KESKIN, N. & GÜNER, A. 1999. Biodegradation of polyvinylchloride (PVC) by white rot fungi. *Bulletin of environmental contamination and toxicology*, 63, 335-342.
- KIRCHMAN, D. L. 2008. New light on an important microbe in the ocean. *Proceedings of the National Academy of Sciences*, 105, 8487-8488.
- KIRCHMAN, D. L., SUZUKI, Y., GARSIDE, C. & DUCKLOW, H. W. 1991. High turnover rates of dissolved organic carbon during a spring phytoplankton bloom. *Nature*, 352, 612.
- KIRSTEIN, I. V., WICHELS, A., KROHNE, G. & GERDTS, G. 2018. Mature biofilm communities on synthetic polymers in seawater-Specific or general? *Marine environmental research*, 142, 147-154.
- KITADA, Y., KAWAHATA, H., SUZUKI, A. & OOMORI, T. 2008. Distribution of pesticides and bisphenol A in sediments collected from rivers adjacent to coral reefs. *Chemosphere*, 71, 2082-2090.
- KLEIN, S., DIMZON, I. K., EUBELER, J. & KNEPPER, T. P. 2018. Analysis, Occurrence, and Degradation of Microplastics in the Aqueous Environment. In: WAGNER, M. & LAMBERT, S. (eds.) *Freshwater Microplastics : Emerging Environmental Contaminants?* Cham: Springer International Publishing.
- KOELMANS, A. A., BESSELING, E., WEGNER, A. & FOEKEMA, E. M. 2013. Plastic as a Carrier of POPs to Aquatic Organisms: A Model Analysis. *Environmental Science & Technology*, 47, 7812-7820.
- KOOI, M., BESSELING, E., KROEZE, C., VAN WEZEL, A. P. & KOELMANS, A. A. 2018. Modeling the Fate and Transport of Plastic Debris in Freshwaters: Review and Guidance. In: WAGNER, M. & LAMBERT, S. (eds.) *Freshwater Microplastics : Emerging Environmental Contaminants?* Cham: Springer International Publishing.

- KOOI, M., NES, E. H. V., SCHEFFER, M. & KOELMANS, A. A. 2017. Ups and downs in the ocean: effects of biofouling on vertical transport of microplastics. *Environmental science & technology*, 51, 7963-7971.
- KOOP, T., LUO, B., TSIAS, A. & PETER, T. 2000. Water activity as the determinant for homogeneous ice nucleation in aqueous solutions. *Nature*, 406, 611.
- KOSTKA, J. E., PRAKASH, O., OVERHOLT, W. A., GREEN, S., FREYER, G., CANION, A., DELGARDIO, J., NORTON, N., HAZEN, T. C. & HUETTEL, M. 2011. Hydrocarbon-degrading bacteria and the bacterial community response in Gulf of Mexico beach sands impacted by the Deepwater Horizon oil spill. *Applied and environmental microbiology*, AEM. 05402-11.
- KOUZUMA, A., KATO, S. & WATANABE, K. 2015. Microbial interspecies interactions: recent findings in syntrophic consortia. *Frontiers in microbiology*, 6, 477.
- KOZICH, J. J., WESTCOTT, S. L., BAXTER, N. T., HIGHLANDER, S. K. & SCHLOSS, P. D. 2013. Development of a Dual-Index Sequencing Strategy and Curation Pipeline for Analyzing Amplicon Sequence Data on the MiSeq Illumina Sequencing Platform. *Applied and Environmental Microbiology*, 79, 5112-5120.
- KRAUSE, E., WICHELS, A., GIMÉNEZ, L., LUNAU, M., SCHILHABEL, M. B. & GERDTS, G. 2012. Small changes in pH have direct effects on marine bacterial community composition: a microcosm approach. *PloS one*, 7, e47035.
- KRUEGER, M. C., HARMS, H. & SCHLOSSER, D. 2015. Prospects for microbiological solutions to environmental pollution with plastics. *Applied Microbiology and Biotechnology*, 99, 8857-8874.
- KU, T.-H. & LIN, C.-A. 2005. Rheological properties of thermoplastic polyvinyl alcohol and polypropylene blend melts in capillary extrusions. *Journal of Polymer Research*, 12, 23-29.
- KÜHN, S., BRAVO REBOLLEDO, E. L. & VAN FRANEKER, J. A. 2015. Deleterious Effects of Litter on Marine Life. In: BERGMANN, M., GUTOW, L. & KLAGES, M. (eds.) *Marine Anthropogenic Litter*. Cham: Springer International Publishing.
- KUJAWINSKI, E. B. 2011. The Impact of Microbial Metabolism on Marine Dissolved Organic Matter. *Annual Review of Marine Science*, 3, 567-599.
- KUKULKA, T., PROSKUROWSKI, G., MORÉT-FERGUSON, S., MEYER, D. W. & LAW, K. L. 2012. The effect of wind mixing on the vertical distribution of buoyant plastic debris. *Geophysical Research Letters*, 39.
- KUMARI, A., CHAUDHARY, D. R. & JHA, B. 2019. Destabilization of polyethylene and polyvinylchloride structure by marine bacterial strain. *Environmental Science and Pollution Research*, 26, 1507-1516.
- LACOSTE, J. & CARLSSON, D. 1992. Gamma-, photo-, and thermally-initiated oxidation of linear low density polyethylene: A quantitative comparison of oxidation products. *Journal of Polymer Science Part A: Polymer Chemistry*, 30, 493-500.
- LACZI, K., KIS, Á., HORVÁTH, B., MARÓTI, G., HEGEDÜS, B., PEREI, K. & RÁKHELY, G. 2015. Metabolic responses of *Rhodococcus erythropolis* PR4 grown

- on diesel oil and various hydrocarbons. *Applied Microbiology and Biotechnology*, 99, 9745-9759.
- LAGE, O. M. & BONDOSO, J. 2014. Planctomycetes and macroalgae, a striking association. *Frontiers in microbiology*, 5, 267.
- LAGIER, J. C., ARMOUGOM, F., MILLION, M., HUGON, P., PAGNIER, I., ROBERT, C., BITTAR, F., FOURNOUS, G., GIMENEZ, G., MARANINCHI, M., TRAPE, J. F., KOONIN, E. V., LA SCOLA, B. & RAOULT, D. 2012. Microbial culturomics: paradigm shift in the human gut microbiome study. *Clinical Microbiology and Infection*, 18, 1185-1193.
- LAMBERT, S. & WAGNER, M. 2016. Formation of microscopic particles during the degradation of different polymers. *Chemosphere*, 161, 510-517.
- LANE, D. J. 1991. 16S/23S rRNA sequencing. In: STACKEBRANDT E, G. M. (ed.) *Nucleic acid techniques in bacterial systematics*. John Wiley & Sons, New York, N.Y., pp. 115-175.
- LANGENHEDER, S., LINDSTRÖM, E. S. & TRANVIK, L. J. 2005. Weak coupling between community composition and functioning of aquatic bacteria. *Limnology and Oceanography*, 50, 957-967.
- LAW, K. L. 2017. Plastics in the Marine Environment. *Annual Review of Marine Science*, 9, 205-229.
- LE BORGNE, S., PANIAGUA, D. & VAZQUEZ-DUHALT, R. 2008. Biodegradation of organic pollutants by halophilic bacteria and archaea. *Journal of molecular microbiology and biotechnology*, 15, 74-92.
- LE DIGABEL, F. & AVÉROUS, L. 2006. Effects of lignin content on the properties of lignocellulose-based biocomposites. *Carbohydrate Polymers*, 66, 537-545.
- LEATHERS, T. D., GOVIND, N. S. & GREENE, R. V. 2000. Biodegradation of Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) by a Tropical Marine Bacterium, *Pseudoalteromonas* sp. NRRL B-30083. *Journal of Polymers and the Environment*, 8, 119-124.
- LEE, J.-W., NAM, J.-H., KIM, Y.-H., LEE, K.-H. & LEE, D.-H. 2008. Bacterial communities in the initial stage of marine biofilm formation on artificial surfaces. *The Journal of Microbiology*, 46, 174-182.
- LEE, Y. M., CHO, K. H., HWANG, K., KIM, E. H., KIM, M., HONG, S. G. & LEE, H. K. 2016. Succession of bacterial community structure during the early stage of biofilm development in the Antarctic marine environment. *The Korean Journal of Microbiology*, 52, 49-58.
- LEGENDRE, L. & LE FÈVRE, J. 1995. Microbial food webs and the export of biogenic carbon in oceans. *Aquatic Microbial Ecology*, 9, 69-77.
- LEONAS, K. K. 1993. The disintegration rate of traditional and chemically modified plastic films in simulated fresh- and sea-water environments. *Journal of Applied Polymer Science*, 47, 2103-2110.

- LI, W. C., TSE, H. F. & FOK, L. 2016. Plastic waste in the marine environment: A review of sources, occurrence and effects. *Science of The Total Environment*, 566-567, 333-349.
- LI, Z., FREDERICKS, P. M., RINTOUL, L. & WARD, C. R. 2007. Application of attenuated total reflectance micro-Fourier transform infrared (ATR-FTIR) spectroscopy to the study of coal macerals: Examples from the Bowen Basin, Australia. *International Journal of Coal Geology*, 70, 87-94.
- LILY, M. K., BAHUGUNA, A., DANGWAL, K. & GARG, V. 2009. Degradation of Benzo [a] Pyrene by a novel strain *Bacillus subtilis* BMT4i (MTCC 9447). *Brazilian journal of microbiology : [publication of the Brazilian Society for Microbiology]*, 40, 884-892.
- LINDH, M. V., LEFÉBURE, R., DEGERMAN, R., LUNDIN, D., ANDERSSON, A. & PINHASSI, J. 2015. Consequences of increased terrestrial dissolved organic matter and temperature on bacterioplankton community composition during a Baltic Sea mesocosm experiment. *AMBIO*, 44, 402-412.
- LITHNER, D., LARSSON, Å. & DAVE, G. 2011. Environmental and health hazard ranking and assessment of plastic polymers based on chemical composition. *Science of The Total Environment*, 409, 3309-3324.
- LIU, J., WEINBAUER, M. G., MAIER, C., DAI, M. & GATTUSO, J.-P. 2010. Effect of ocean acidification on microbial diversity and on microbe-driven biogeochemistry and ecosystem functioning. *Aquatic Microbial Ecology*, 61, 291-305.
- LIU, W.-T., MARSH, T. L., CHENG, H. & FORNEY, L. J. 1997. Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. *Appl Environ Microbiol*, 63.
- LOBELLE, D. & CUNLIFFE, M. 2011. Early microbial biofilm formation on marine plastic debris. *Marine Pollution Bulletin*, 62, 197-200.
- LOEB, G. I. & NEIHOF, R. A. 1975. Marine Conditioning Films. *Applied Chemistry at Protein Interfaces*. AMERICAN CHEMICAL SOCIETY.
- LÓPEZ-PÉREZ, M., GONZAGA, A., MARTIN-CUADRADO, A.-B., ONYSHCHENKO, O., GHAVIDEL, A., GHAI, R. & RODRIGUEZ-VALERA, F. 2012. Genomes of surface isolates of *Alteromonas macleodii*: the life of a widespread marine opportunistic copiotroph. *Scientific reports*, 2, 696.
- LUCAS, N., BIENAIME, C., BELLOY, C., QUENEUDEC, M., SILVESTRE, F. & NAVA-SAUCEDO, J.-E. 2008. Polymer biodegradation: Mechanisms and estimation techniques – A review. *Chemosphere*, 73, 429-442.
- LURIA, C. M., AMARAL-ZETTLER, L. A., DUCKLOW, H. W., REPETA, D. J., RHYNE, A. L. & RICH, J. J. 2017. Seasonal shifts in bacterial community responses to phytoplankton-derived dissolved organic matter in the Western Antarctic Peninsula. *Frontiers in microbiology*, 8, 2117.
- MAEKAWA, Y., SUZUKI, Y., MAEYAMA, K., YONEZAWA, N. & YOSHIDA, M. 2006. Chemical modification of the internal surfaces of cylindrical pores of submicrometer size in poly (ethylene terephthalate). *Langmuir*, 22, 2832-2837.

- MAGNUSSON, K. & NORÉN, F. 2014. Screening of microplastic particles in and downstream a wastewater treatment plant. *IVL Report C*, 55, 22.
- MARCHESI, J. R., SATO, T., WEIGHTMAN, A. J., MARTIN, T. A., FRY, J. C., HIOM, S. J. & WADE, W. G. 1998. Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. *Appl Environ Microbiol*, 64.
- MARIE, D., PARTENSKY, F., JACQUET, S. & VAULOT, D. 1997. Enumeration and cell cycle analysis of natural populations of marine picoplankton by flow cytometry using the nucleic acid stain SYBR Green I. *Applied and environmental microbiology*, 63, 186-193.
- MARTENS, T., GRAM, L., GROSSART, H.-P., KESSLER, D., MÜLLER, R., SIMON, M., WENZEL, S. C. & BRINKHOFF, T. 2007. Bacteria of the Roseobacter clade show potential for secondary metabolite production. *Microbial ecology*, 54, 31-42.
- MARTIN, O., ASA, M., KAROLINA, I., EVA, H., ZANDRA, G., I. U. K., ZOLTAN, B. & ELENA, G. 2018. Evidence for selective bacterial community structuring on microplastics. *Environmental Microbiology*, 0.
- MARTINY, A. C., ALBRECHTSEN, H.-J., ARVIN, E. & MOLIN, S. 2005. Identification of Bacteria in Biofilm and Bulk Water Samples from a Nonchlorinated Model Drinking Water Distribution System: Detection of a Large Nitrite-Oxidizing Population Associated with *Nitrospira* spp. *Applied and Environmental Microbiology*, 71, 8611.
- MARTINY, A. C., JØRGENSEN, T. M., ALBRECHTSEN, H.-J., ARVIN, E. & MOLIN, S. 2003. Long-term succession of structure and diversity of a biofilm formed in a model drinking water distribution system. *Appl. Environ. Microbiol.*, 69, 6899-6907.
- MATSUMURA, S., TOMIZAWA, N., TOKI, A., NISHIKAWA, K. & TOSHIMA, K. 1999. Novel poly (vinyl alcohol)-degrading enzyme and the degradation mechanism. *Macromolecules*, 32, 7753-7761.
- MCILGORM, A., CAMPBELL, H. F. & RULE, M. J. 2011. The economic cost and control of marine debris damage in the Asia-Pacific region. *Ocean & Coastal Management*, 54, 643-651.
- MCKEEN, L. W. 2008. 1 - Introduction to Plastics and Elastomers. In: MCKEEN, L. W. (ed.) *Effect of Temperature and other Factors on Plastics and Elastomers (Second Edition)*. Norwich, NY: William Andrew Publishing.
- MEENAKUMARI, B., RADHALEKSHMY, K. & PANICKER, P. 1995. Weathering of nylon netting yarns. *Fishery Technology*, 32, 85-88.
- MERGAERT, J., WOUTERS, A., SWINGS, J. & ANDERSON, C. 1995. In situ biodegradation of poly (3-hydroxybutyrate) and poly (3-hydroxybutyrate-co-3-hydroxyvalerate) in natural waters. *Canadian Journal of Microbiology*, 41, 154-159.
- MERON, D., ATIAS, E., KRUH, L. I., ELIFANTZ, H., MINZ, D., FINE, M. & BANIN, E. 2011. The impact of reduced pH on the microbial community of the coral *Acropora eurystoma*. *The ISME journal*, 5, 51.

- MERON, D., RODOLFO-METALPA, R., CUNNING, R., BAKER, A. C., FINE, M. & BANIN, E. 2012. Changes in coral microbial communities in response to a natural pH gradient. *The ISME journal*, 6, 1775.
- MEYER, F. W., VOGEL, N., DIELE, K., KUNZMANN, A., UTHICKE, S. & WILD, C. 2016. Effects of High Dissolved Inorganic and Organic Carbon Availability on the Physiology of the Hard Coral *Acropora millepora* from the Great Barrier Reef. *PloS one*, 11, e0149598.
- MIN, K., CUIFFI, J. D. & MATHERS, R. T. 2020. Ranking environmental degradation trends of plastic marine debris based on physical properties and molecular structure. *Nature communications*, 11, 1-11.
- MOHAN, K. 2011. Microbial deterioration and degradation of polymeric materials. *Journal of Biochemical Technology*, 2, 210-215.
- MOHANRASU, K., PREMNATH, N., SIVA PRAKASH, G., SUDHAKAR, M., BOOBALAN, T. & ARUN, A. 2018. Exploring multi potential uses of marine bacteria; an integrated approach for PHB production, PAHs and polyethylene biodegradation. *Journal of Photochemistry and Photobiology B: Biology*, 185, 55-65.
- MOJTAHID, M., JORISSEN, F. & PEARSON, T. H. 2008. Comparison of benthic foraminiferal and macrofaunal responses to organic pollution in the Firth of Clyde (Scotland). *Marine Pollution Bulletin*, 56, 42-76.
- MORAN, M. A., SATINSKY, B., GIFFORD, S. M., LUO, H., RIVERS, A., CHAN, L.-K., MENG, J., DURHAM, B. P., SHEN, C., VARALJAY, V. A., SMITH, C. B., YAGER, P. L. & HOPKINSON, B. M. 2013. Sizing up metatranscriptomics. *The ISME Journal*, 7, 237-243.
- MORÉ-FERGUSON, S., LAW, K. L., PROSKUROWSKI, G., MURPHY, E. K., PEACOCK, E. E. & REDDY, C. M. 2010. The size, mass, and composition of plastic debris in the western North Atlantic Ocean. *Marine Pollution Bulletin*, 60, 1873-1878.
- MOUAT, J., LOZANO, R. L. & BATESON, H. 2010. Economic Impacts of Marine Litter. *Kommunenenes Internasjonale Miljøorganisasjon (KIMO): Grantfield*, [http://www.kimointernational.org/wp/wp-content/uploads/2017/09/KIMO\\_Economic-Impacts-of-Marine-Litter.pdf](http://www.kimointernational.org/wp/wp-content/uploads/2017/09/KIMO_Economic-Impacts-of-Marine-Litter.pdf).
- MULLER, R. 2005. Handbook of biodegradable polymers. *Rapra Technology Limited, UK*, 303-337.
- MUYZER, G., DE WAAL, E. C. & UITTERLINDEN, A. G. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and environmental microbiology*, 59, 695-700.
- NAKAMURA, H., NAKAMURA, T., NOGUCHI, T. & IMAGAWA, K. 2006. Photodegradation of PEEK sheets under tensile stress. *Polymer Degradation and Stability*, 91, 740-746.

- NAUENDORF, A., KRAUSE, S., BIGALKE, N. K., GORB, E. V., GORB, S. N., HAECKEL, M., WAHL, M. & TREUDE, T. 2016. Microbial colonization and degradation of polyethylene and biodegradable plastic bags in temperate fine-grained organic-rich marine sediments. *Marine pollution bulletin*, 103, 168-178.
- NEWMAN, S., WATKINS, E., FARMER, A., BRINK, P. T. & SCHWEITZER, J.-P. 2015. The Economics of Marine Litter. In: BERGMANN, M., GUTOW, L. & KLAGES, M. (eds.) *Marine Anthropogenic Litter*. Cham: Springer International Publishing.
- NGUYEN-TRI, P. & PRUD'HOMME, R. E. 2019. Nanoscale analysis of the photodegradation of polyester fibers by AFM-IR. *Journal of Photochemistry and Photobiology A: Chemistry*, 371, 196-204.
- NIAOUNAKIS, M. 2017. 3 - Degradation of Plastics in the Marine Environment. In: NIAOUNAKIS, M. (ed.) *Management of Marine Plastic Debris*. William Andrew Publishing.
- NICHOLS, P. D., MICHAEL HENSON, J., GUCKERT, J. B., NIVENS, D. E. & WHITE, D. C. 1985. Fourier transform-infrared spectroscopic methods for microbial ecology: analysis of bacteria, bacteri-polymer mixtures and biofilms. *Journal of Microbiological Methods*, 4, 79-94.
- NISHIKIDA, K. & COATES, J. 2003. Infrared and Raman analysis of polymers. In: LOBO, H. & BONILLA, J. V. (eds.) *Handbook of plastics analysis*. Marcel Dekker, Inc, New York, USA, pp. 206-345.
- NODA, I., DOWREY, A. E., HAYNES, J. L. & MARCOTT, C. 2007. Group Frequency Assignments for Major Infrared Bands Observed in Common Synthetic Polymers. In: MARK, J. E. (ed.) *Physical Properties of Polymers Handbook*. Springer New York, USA, pp. 395-406.
- NOGALES, B., LANFRANCONI, M. P., PIÑA-VILLALONGA, J. M. & BOSCH, R. 2011. Anthropogenic perturbations in marine microbial communities. *FEMS Microbiology Reviews*, 35, 275-298.
- NOGI, Y., YOSHIKUMI, M. & MIYAZAKI, M. 2014. *Thalassospira povalilytica* sp. nov., a polyvinyl-alcohol-degrading marine bacterium. *International journal of systematic and evolutionary microbiology*, 64, 1149-1153.
- O'BRINE, T. & THOMPSON, R. C. 2010. Degradation of plastic carrier bags in the marine environment. *Mar Pollut Bull*, 60, 2279-83.
- OBERBECKMANN, S., KREIKEMEYER, B. & LABRENZ, M. 2018. Environmental Factors Support the Formation of Specific Bacterial Assemblages on Microplastics. *Frontiers in Microbiology*, 8.
- OBERBECKMANN, S., LÖDER, M., GERDTS, G. & OSBORN, A. M. 2014. Spatial and seasonal variation in diversity and structure of microbial biofilms on marine plastics in Northern European waters. *FEMS Microbiology Ecology*, 90, 478-492.
- OBERBECKMANN, S., OSBORN, A. M. & DUHAIME, M. B. 2016. Microbes on a Bottle: Substrate, Season and Geography Influence Community Composition of Microbes Colonizing Marine Plastic Debris. *PLOS ONE*, 11, e0159289.



- OJEDA, J. J., ROMERO-GONZÁLEZ, M. E. & BANWART, S. A. 2009. Analysis of Bacteria on Steel Surfaces Using Reflectance Micro-Fourier Transform Infrared Spectroscopy. *Analytical Chemistry*, 81, 6467-6473.
- OLAYAN, H. B., HAMI, H. S. & OWEN, E. D. 1996. Photochemical and Thermal Crosslinking of Polymers. *Journal of Macromolecular Science, Part C*, 36, 671-719.
- OLIANI, W. L., PARRA, D. F., OTAGURO, H., LIMA, L. F. & LUGÃO, A. B. 2007. Study of the weathering of high melt strength polypropylene (HMS-PP). *International Nuclear Atlantic Conference (INAC 2007)*, 31.
- OLIVEIRA, M., RIBEIRO, A., HYLLAND, K. & GUILHERMINO, L. 2013. Single and combined effects of microplastics and pyrene on juveniles (0+ group) of the common goby *Pomatoschistus microps* (Teleostei, Gobiidae). *Ecological Indicators*, 34, 641-647.
- ORR, G. I., HADAR, Y. & SIVAN, A. 2004a. Colonization, biofilm formation and biodegradation of polyethylene by a strain of *Rhodococcus ruber*. *Applied Microbiology and Biotechnology*, 65, 97-104.
- ORR, I. G., HADAR, Y. & SIVAN, A. 2004b. Colonization, biofilm formation and biodegradation of polyethylene by a strain of *Rhodococcus ruber*. *Applied Microbiology and Biotechnology*, 65, 97-104.
- OSMAN, Y. A., ELRAZAK, A. A., KHATER, W., NASHY, E.-S. & MOHAMADEEN, A. 2015. Molecular Characterization of a Poly- $\beta$ -Hydroxybutyrate-Producing Microbacterium Isolate. *International Journal of Applied Sciences and Biotechnology*, 3, 143-150.
- PAÇO, A., DUARTE, K., DA COSTA, J. P., SANTOS, P. S. M., PEREIRA, R., PEREIRA, M. E., FREITAS, A. C., DUARTE, A. C. & ROCHA-SANTOS, T. A. P. 2017. Biodegradation of polyethylene microplastics by the marine fungus *Zalerion maritimum*. *Science of The Total Environment*, 586, 10-15.
- PALMISANO, A. C. & PETTIGREW, C. A. 1992. Biodegradability of Plastics. *BioScience*, 42, 680-685.
- PAPA, R., PARRILLI, E. & SANNIA, G. 2009. Engineered marine Antarctic bacterium *Pseudoalteromonas haloplanktis* TAC125: a promising micro-organism for the bioremediation of aromatic compounds. *Journal of Applied Microbiology*, 106, 49-56.
- PARK, H. Y., KIM, K. K., JIN, L. & LEE, S.-T. 2006a. *Microbacterium paludicola* sp. nov., a novel xylanolytic bacterium isolated from swamp forest. *International Journal of Systematic and Evolutionary Microbiology*, 56, 535-539.
- PARK, S.-J., KANG, C.-H., NAM, Y.-D., BAE, J.-W., PARK, Y.-H., QUAN, Z.-X., MOON, D.-S., KIM, H.-J., ROH, D.-H. & RHEE, S.-K. 2006b. *Oceanisphaera donghaensis* sp. nov., a halophilic bacterium from the East Sea, Korea. *International Journal of Systematic and Evolutionary Microbiology*, 56, 895-898.
- PARK, S., OH, K.-H., LEE, S.-Y., OH, T.-K. & YOON, J.-H. 2012. *Cellulophaga geojensis* sp. nov., a member of the family Flavobacteriaceae isolated from marine sand. *International Journal of Systematic and Evolutionary Microbiology*, 62, 1354-1358.

- PARRISH, K. & FAHRENFELD, N. L. 2019. Microplastic biofilm in fresh- and wastewater as a function of microparticle type and size class. *Environmental Science: Water Research & Technology*, 5, 495-505.
- PATEL-SORRENTINO, N., MOUNIER, S., LUCAS, Y. & BENAÏM, J.-Y. 2004. Effects of UV–visible irradiation on natural organic matter from the Amazon basin. *Science of the total Environment*, 321, 231-239.
- PAULI, N.-C., PETERMANN, J. S., LOTT, C. & WEBER, M. 2017. Macrofouling communities and the degradation of plastic bags in the sea: an *in situ* experiment. *Royal Society Open Science*, 4, 170549.
- PAVIA, D. L., LAMPMAN, G. M., KRIZ, G. S. & VYVYAN, J. A. 2008. *Introduction to spectroscopy (4<sup>th</sup> Ed.)*, Cengage Learning, Belmont, CA, USA, 52-58pp.
- PEARSON, P. N. & PALMER, M. R. 2000. Atmospheric carbon dioxide concentrations over the past 60 million years. *Nature*, 406, 695.
- PEGRAM, J. E. & ANDRADY, A. L. 1989. Outdoor weathering of selected polymeric materials under marine exposure conditions. *Polymer Degradation and Stability*, 26, 333-345.
- PETERS, S. J. & EWING, G. E. 1997. Water on Salt: An Infrared Study of Adsorbed H<sub>2</sub>O on NaCl(100) under Ambient Conditions. *The Journal of Physical Chemistry B*, 101, 10880-10886.
- PETROVA, O. E. & SAUER, K. 2012. Sticky Situations: Key Components That Control Bacterial Surface Attachment. *Journal of Bacteriology*, 194, 2413.
- PIELICHOWSKI, K. & NJUGUNA, J. 2005. Thermal Degradation of Polymeric Materials. Smithers Rapra Technology.
- PIRZADEH, E., ZADHOUSH, A. & HAGHIGHAT, M. 2007. Hydrolytic and thermal degradation of PET fibers and PET granule: The effects of crystallization, temperature, and humidity. *Journal of applied polymer science*, 106, 1544-1549.
- PLASTICS EUROPE 2018. Plastics—The Facts 2018 *An analysis of European plastics production, demand and waste data*, [https://www.plasticseurope.org/application/files/6315/4510/9658/Plastics\\_the\\_facts\\_2018\\_AF\\_web.pdf](https://www.plasticseurope.org/application/files/6315/4510/9658/Plastics_the_facts_2018_AF_web.pdf).
- PLOT, V. & GEORGES, J.-Y. 2010. Plastic Debris in a Nesting Leatherback Turtle in French Guiana. *Chelonian Conservation and Biology*, 9, 267-270.
- POLLET, T., BERDJEB, L., GARNIER, C., DURRIEU, G., LE POUPON, C., MISSON, B. & JEAN-FRANÇOIS, B. 2018. Prokaryotic community successions and interactions in marine biofilms: the key role of Flavobacteriia. *FEMS microbiology ecology*, 94, fty083.
- POMEROY, L. R., LEB. WILLIAMS, P. J., AZAM, F. & HOBBIIE, J. E. 2007. The microbial loop. *Oceanography*, 20, 28-33.

- PRAMILA, R. & RAMESH, K. V. 2011. Biodegradation of low density polyethylene (LDPE) by fungi isolated from marine water a SEM analysis. *African Journal of Microbiology Research*, 5, 5013-5018.
- PROVENCHER, J. F., BOND, A. L., AVERY-GOMM, S., BORRELLE, S. B., BRAVO REBOLLEDO, E. L., HAMMER, S., KÜHN, S., LAVERS, J. L., MALLORY, M. L., TREVAIL, A. & VAN FRANEKER, J. A. 2017. Quantifying ingested debris in marine megafauna: a review and recommendations for standardization. *Analytical Methods*, 9, 1454-1469.
- QUAST, C., PRUESSE, E., YILMAZ, P., GERKEN, J., SCHWEER, T., YARZA, P., PEPLIES, J. & GLÖCKNER, F. O. 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Research*, 41, D590-D596.
- RAGHUL, S. S., BHAT, S. G., CHANDRASEKARAN, M., FRANCIS, V. & THACHIL, E. T. 2014. Biodegradation of polyvinyl alcohol-low linear density polyethylene-blended plastic film by consortium of marine benthic vibrios. *International Journal of Environmental Science and Technology*, 11, 1827-1834.
- RAJEEV, M., SUSHMITHA, T. J., TOLETI, S. R. & PANDIAN, S. K. 2019. Culture dependent and independent analysis and appraisal of early stage biofilm-forming bacterial community composition in the Southern coastal seawater of India. *Science of The Total Environment*, 666, 308-320.
- RAMETTE, A. 2007. Multivariate analyses in microbial ecology. *FEMS microbiology ecology*, 62, 142-160.
- RAY, S. & COONEY, R. P. 2018. Thermal degradation of polymer and polymer composites. *Handbook of environmental degradation of materials*. William Andrew Publishing, pp. 185-206.
- REISSER, J., SHAW, J., HALLEGRAEFF, G., PROIETTI, M., BARNES, D. K. A., THUMS, M., WILCOX, C., HARDESTY, B. D. & PATTIARATCHI, C. 2014. Millimeter-sized marine plastics: a new pelagic habitat for microorganisms and invertebrates. *PLoS One*, 9, e100289.
- RENDUELES, O. & GHIGO, J.-M. 2012. Multi-species biofilms: how to avoid unfriendly neighbors. *FEMS Microbiology Reviews*, 36, 972-989.
- RESTREPO-FLÓREZ, J.-M., BASSI, A. & THOMPSON, M. R. 2014. Microbial degradation and deterioration of polyethylene—A review. *International Biodeterioration & Biodegradation*, 88, 83-90.
- RICKARD, A. H., GILBERT, P., HIGH, N. J., KOLENBRANDER, P. E. & HANDLEY, P. S. 2003. Bacterial coaggregation: an integral process in the development of multi-species biofilms. *Trends in Microbiology*, 11, 94-100.
- ROCHMAN, C. M. 2015. The Complex Mixture, Fate and Toxicity of Chemicals Associated with Plastic Debris in the Marine Environment. In: BERGMANN, M., GUTOW, L. & KLAGES, M. (eds.) *Marine Anthropogenic Litter*. Cham: Springer International Publishing.

- ROCHMAN, C. M., HOH, E., HENTSCHEL, B. T. & KAYE, S. 2013a. Long-term field measurement of sorption of organic contaminants to five types of plastic pellets: implications for plastic marine debris. *Environmental Science & Technology*, 47, 1646-1654.
- ROCHMAN, C. M., HOH, E., KUROBE, T. & TEH, S. J. 2013b. Ingested plastic transfers hazardous chemicals to fish and induces hepatic stress. *Scientific Reports*, 3, 3263.
- ROGNES, T., FLOURI, T., NICHOLS, B., QUINCE, C. & MAHÉ, F. 2016. VSEARCH: a versatile open source tool for metagenomics. *PeerJ*, 4, e2584.
- ROMERA-CASTILLO, C., LETSCHER, R. T. & HANSELL, D. A. 2016. New nutrients exert fundamental control on dissolved organic carbon accumulation in the surface Atlantic Ocean. *Proceedings of the National Academy of Sciences*, 113, 10497-10502.
- ROMERA-CASTILLO, C., PINTO, M., LANGER, T. M., ÁLVAREZ-SALGADO, X. A. & HERNDL, G. J. 2018. Dissolved organic carbon leaching from plastics stimulates microbial activity in the ocean. *Nature Communications*, 9, 1430.
- RONKVIST, Å. M., XIE, W., LU, W. & GROSS, R. A. 2009. Cutinase-catalyzed hydrolysis of poly (ethylene terephthalate). *Macromolecules*, 42, 5128-5138.
- RUMMEL, C. D., JAHNKE, A., GOROKHOVA, E., KÜHNEL, D. & SCHMITT-JANSEN, M. 2017. Impacts of biofilm formation on the fate and potential effects of microplastic in the aquatic environment. *Environmental Science & Technology Letters*, 4, 258-267.
- RYAN, P. G. 2015. A Brief History of Marine Litter Research. In: BERGMANN, M., GUTOW, L. & KLAGES, M. (eds.) *Marine Anthropogenic Litter*. Cham: Springer International Publishing.
- SAH, A., NEGI, H., KAPRI, A., ANWAR, S. & GOEL, R. 2011. Comparative shelf life and efficacy of LDPE and PVC degrading bacterial consortia under bioformulation. *Ekologija*, 57.
- SAKAI, K., FUKUBA, M., HASUI, Y., MORIYOSHI, K., OHMOTO, T., FUJITA, T. & OHE, T. 1998. Purification and characterization of an esterase involved in poly (vinyl alcohol) degradation by *Pseudomonas vesicularis* PD. *Bioscience, biotechnology, and biochemistry*, 62, 2000-2007.
- SALTA, M., WHARTON, J. A., BLACHE, Y., STOKES, K. R. & BRIAND, J. F. 2013. Marine biofilms on artificial surfaces: structure and dynamics. *Environmental microbiology*, 15, 2879-2893.
- SANDERS, E. R. 2012. Aseptic laboratory techniques: plating methods. *Journal of visualized experiments : JoVE*, e3064-e3064.
- SANGALE, M., SHAHNAWAZ, M. & ADE, A. 2012. A review on biodegradation of polythene: the microbial approach. *J Bioremed Biodeg*, 3, 1-9.
- SANGEETHA DEVI, R., RAMYA, R., KANNAN, K., ROBERT ANTONY, A. & RAJESH KANNAN, V. 2019. Investigation of biodegradation potentials of high

density polyethylene degrading marine bacteria isolated from the coastal regions of Tamil Nadu, India. *Marine Pollution Bulletin*, 138, 549-560.

SANGWAN, P., WAY, C. & WU, D. Y. 2009. New insight into biodegradation of polylactide (PLA)/clay nanocomposites using molecular ecological techniques. *Macromolecular bioscience*, 9, 677-686.

SANGWAN, P. & WU, D. Y. 2008. New insights into polylactide biodegradation from molecular ecological techniques. *Macromolecular bioscience*, 8, 304-315.

SASHIWA, H., FUKUDA, R., OKURA, T., SATO, S. & NAKAYAMA, A. 2018. Microbial Degradation Behavior in Seawater of Polyester Blends Containing Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHHx). *Marine Drugs*, 16, 34.

SAVOCA, M. S., WOHLFEIL, M. E., EBELER, S. E. & NEVITT, G. A. 2016. Marine plastic debris emits a keystone infochemical for olfactory foraging seabirds. *Science Advances*, 2, e1600395.

SCHLOSS, P. D., WESTCOTT, S. L., RYABIN, T., HALL, J. R., HARTMANN, M., HOLLISTER, E. B., LESNIEWSKI, R. A., OAKLEY, B. B., PARKS, D. H., ROBINSON, C. J., SAHL, J. W., STRES, B., THALLINGER, G. G., VAN HORN, D. J. & WEBER, C. F. 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl Environ Microbiol*, 75.

SCHUYLER, Q. A., WILCOX, C., TOWNSEND, K., HARDESTY, B. D. & MARSHALL, N. J. 2014. Mistaken identity? Visual similarities of marine debris to natural prey items of sea turtles. *BMC Ecology*, 14, 14.

SCOTT, G. 1976. Time controlled stabilization of polyolefins. *Journal of Polymer Science: Polymer Symposia*, 57, 357-374.

SEEKELL, D. A., LAPIERRE, J. F., ASK, J., BERGSTRÖM, A. K., DEININGER, A., RODRÍGUEZ, P. & KARLSSON, J. 2015. The influence of dissolved organic carbon on primary production in northern lakes. *Limnology and Oceanography*, 60, 1276-1285.

SEELEY, M. E., SONG, B., PASSIE, R. & HALE, R. C. 2020. Microplastics affect sedimentary microbial communities and nitrogen cycling. *Nature Communications*, 11, 2372.

SEKIGUCHI, T., EBISUI, A., NOMURA, K., WATANABE, T., ENOKI, M. & KANEHIRO, H. 2009. Biodegradation of several fibers submerged in deep sea water and isolation of biodegradable plastic degrading bacteria from deep ocean water. *NIPPON SUISAN GAKKAISHI*, 75, 1011-1018.

SEKIGUCHI, T., SAIKA, A., NOMURA, K., WATANABE, T., WATANABE, T., FUJIMOTO, Y., ENOKI, M., SATO, T., KATO, C. & KANEHIRO, H. 2011a. Biodegradation of aliphatic polyesters soaked in deep seawaters and isolation of poly( $\epsilon$ -caprolactone)-degrading bacteria. *Polymer Degradation and Stability*, 96, 1397-1403.

SEKIGUCHI, T., SATO, T., ENOKI, M., KANEHIRO, H., UEMATSU, K. & KATO, C. 2011b. Isolation and characterization of biodegradable plastic degrading bacteria

- from deep-sea environments. *JAMSTEC Report of Research and Development*, 11, 33-41.
- SHAH, A. A., HASAN, F., HAMEED, A. & AHMED, S. 2008. Biological degradation of plastics: A comprehensive review. *Biotechnology Advances*, 26, 246-265.
- SHI, W., ZHANG, J., SHI, X.-M. & JIANG, G.-D. 2008. Different photodegradation processes of PVC with different average degrees of polymerization. *Journal of Applied Polymer Science*, 107, 528-540.
- SHIMAO, M. 2001. Biodegradation of plastics. *Current Opinion in Biotechnology*, 12, 242-247.
- SHIN, N.-R., WHON, T. W., ROH, S. W., KIM, M.-S., KIM, Y.-O. & BAE, J.-W. 2012. *Oceanisphaera sediminis* sp. nov., isolated from marine sediment. *International Journal of Systematic and Evolutionary Microbiology*, 62, 1552-1557.
- SIEBURTH, J. M. 1975. *Microbial seascapes : a pictorial essay on marine microorganisms and their environments*, Baltimore, University Park Press.
- SIGNORI, C. N., THOMAS, F., ENRICH-PRAST, A., POLLERY, R. C. & SIEVERT, S. M. 2014. Microbial diversity and community structure across environmental gradients in Bransfield Strait, Western Antarctic Peninsula. *Frontiers in microbiology*, 5, 647.
- SINGH, B. & SHARMA, N. 2008. Mechanistic implications of plastic degradation. *Polymer degradation and stability*, 93, 561-584.
- SIPLER, R. E., KELLOGG, C. T. E., CONNELLY, T. L., ROBERTS, Q. N., YAGER, P. L. & BRONK, D. A. 2017. Microbial Community Response to Terrestrially Derived Dissolved Organic Matter in the Coastal Arctic. *Frontiers in Microbiology*, 8, 1018.
- SIVAN, A., SZANTO, M. & PAVLOV, V. 2006. Biofilm development of the polyethylene-degrading bacterium *Rhodococcus ruber*. *Applied microbiology and biotechnology*, 72, 346-352.
- SJÖSTEDT, J., KOCH-SCHMIDT, P., PONTARP, M., CANBÄCK, B., TUNLID, A., LUNDBERG, P., HAGSTRÖM, Å. & RIEMANN, L. 2012. Recruitment of Members from the Rare Biosphere of Marine Bacterioplankton Communities after an Environmental Disturbance. *Applied and Environmental Microbiology*, 78, 1361-1369.
- SKARIYACHAN, S., SETLUR, A. S., NAIK, S. Y., NAIK, A. A., USHARANI, M. & VASIST, K. S. 2017. Enhanced biodegradation of low and high-density polyethylene by novel bacterial consortia formulated from plastic-contaminated cow dung under thermophilic conditions. *Environmental Science and Pollution Research*, 24, 8443-8457.
- SKOOG, D. A., HOLLER, F. J. & CROUCH, S. R. 2017. *Principles of instrumental analysis* (7<sup>th</sup> Ed.), Cengage learning, Boston, MA, USA, pp. 389-411.
- SMITH, C. J., DANILOWICZ, B. S., CLEAR, A. K., COSTELLO, F. J., WILSON, B. & MEIJER, W. G. 2005. T-Align, a web-based tool for comparison of multiple terminal

restriction fragment length polymorphism profiles. *FEMS microbiology ecology*, 54, 375-380.

SOCRATES, G. 2004. *Infrared and Raman characteristic group frequencies: tables and charts (3<sup>rd</sup> Ed.)*, John Wiley & Sons, Ltd., Chichester, England.

SONG, J. H., MURPHY, R. J., NARAYAN, R. & DAVIES, G. B. H. 2009. Biodegradable and compostable alternatives to conventional plastics. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 364, 2127-2139.

SUDESH, K., ABE, H. & DOI, Y. 2000. Synthesis, structure and properties of polyhydroxyalkanoates: biological polyesters. *Progress in polymer science*, 25, 1503-1555.

SUDHAKAR, M., DOBLE, M., MURTHY, P. S. & VENKATESAN, R. 2008. Marine microbe-mediated biodegradation of low- and high-density polyethylenes. *International Biodeterioration & Biodegradation*, 61, 203-213.

SUH, S.-S., PARK, M., HWANG, J., KIL, E.-J., JUNG, S. W., LEE, S. & LEE, T.-K. 2015. Seasonal dynamics of marine microbial community in the South Sea of Korea. *PLoS One*, 10, e0131633.

SUHRHOFF, T. J. & SCHOLZ-BÖTTCHER, B. M. 2016. Qualitative impact of salinity, UV radiation and turbulence on leaching of organic plastic additives from four common plastics—A lab experiment. *Marine pollution bulletin*, 102, 84-94.

SUMBALI, G. & MEHROTRA, R. 2009. *Growth, culture and maintenance of microorganisms. Principles of microbiology*, Tata McGraw Hill, New Delhi.

SYRANIDOU, E., KARKANORACHAKI, K., AMOROTTI, F., REPOUSKOU, E., KROLL, K., KOLVENBACH, B., CORVINI, P. F. X., FAVA, F. & KALOGERAKIS, N. 2017. Development of tailored indigenous marine consortia for the degradation of naturally weathered polyethylene films. *PLOS ONE*, 12, e0183984.

TALSNESS, C. E., ANDRADE ANDERSON, J. M., KURIYAMA SERGIO, N., TAYLOR JULIA, A. & VOM SAAL FREDERICK, S. 2009. Components of plastic: experimental studies in animals and relevance for human health. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 364, 2079-2096.

TANAKA, K., TAKADA, H., YAMASHITA, R., MIZUKAWA, K., FUKUWAKA, M.-A. & WATANUKI, Y. 2013. Accumulation of plastic-derived chemicals in tissues of seabirds ingesting marine plastics. *Marine Pollution Bulletin*, 69, 219-222.

TANG, C.-C., CHEN, H.-I., BRIMBLECOMBE, P. & LEE, C.-L. 2018. Textural, surface and chemical properties of polyvinyl chloride particles degraded in a simulated environment. *Marine Pollution Bulletin*, 133, 392-401.

TEUTEN, E. L., SAQUING, J. M., KNAPPE, D. R., BARLAZ, M. A., JONSSON, S., BJÖRN, A., ROWLAND, S. J., THOMPSON, R. C., GALLOWAY, T. S. & YAMASHITA, R. 2009. Transport and release of chemicals from plastics to the environment and to wildlife. *Philosophical Transactions of the Royal Society of London B: Biological Sciences*, 364, 2027-2045.

- THINGSTAD, T. F., BELLERBY, R. G. J., BRATBAK, G., BØRSHEIM, K. Y., EGGE, J. K., HELDAL, M., LARSEN, A., NEILL, C., NEJSTGAARD, J., NORLAND, S., SANDAA, R. A., SKJOLDAL, E. F., TANAKA, T., THYRHAUG, R. & TÖPPER, B. 2008. Counterintuitive carbon-to-nutrient coupling in an Arctic pelagic ecosystem. *Nature*, 455, 387-390.
- THOMAS, S. N. & HRIDAYANATHAN, C. 2006. The effect of natural sunlight on the strength of polyamide 6 multifilament and monofilament fishing net materials. *Fisheries Research*, 81, 326-330.
- THOMPSON, F. L., HOSTE, B., VANDEMEULEBROECKE, K. & SWINGS, J. 2001. Genomic Diversity Amongst *Vibrio* Isolates from Different Sources Determined by Fluorescent Amplified Fragment Length Polymorphism. *Systematic and Applied Microbiology*, 24, 520-538.
- THOMPSON, R. C., MOORE, C. J., VOM SAAL, F. S. & SWAN, S. H. 2009. Plastics, the environment and human health: current consensus and future trends. *Philosophical Transactions of the Royal Society of London B: Biological Sciences*, 364, 2153-2166.
- THOMPSON, R. C., OLSEN, Y., MITCHELL, R. P., DAVIS, A., ROWLAND, S. J., JOHN, A. W. G., MCGONIGLE, D. & RUSSELL, A. E. 2004. Lost at sea: where is all the plastic? *Science*, 304.
- TOSIN, M., WEBER, M., SIOTTO, M., LOTT, C. & DEGLI-INNOCENTI, F. 2012. Laboratory Test Methods to Determine the Degradation of Plastics in Marine Environmental Conditions. *Frontiers in Microbiology*, 3, 225.
- TRAVING, S. J., ROWE, O., JAKOBSEN, N. M., SØRENSEN, H., DINASQUET, J., STEDMON, C. A., ANDERSSON, A. & RIEMANN, L. 2017a. The effect of increased loads of dissolved organic matter on estuarine microbial community composition and function. *Frontiers in microbiology*, 8, 351.
- TRAVING, S. J., ROWE, O., JAKOBSEN, N. M., SØRENSEN, H., DINASQUET, J., STEDMON, C. A., ANDERSSON, A. & RIEMANN, L. 2017b. The Effect of Increased Loads of Dissolved Organic Matter on Estuarine Microbial Community Composition and Function. *Frontiers in microbiology*, 8, 351-351.
- TRIBEDI, P., SARKAR, S., MUKHERJEE, K. & SIL, A. K. 2012. Isolation of a novel *Pseudomonas* sp from soil that can efficiently degrade polyethylene succinate. *Environmental Science and Pollution Research*, 19, 2115-2124.
- TSUJI, H. & MIYAUCHI, S. 2001. Poly(l-lactide): VI Effects of crystallinity on enzymatic hydrolysis of poly(l-lactide) without free amorphous region. *Polymer Degradation and Stability*, 71, 415-424.
- TYLER, D. R. 2004. Mechanistic aspects of the effects of stress on the rates of photochemical degradation reactions in polymers. *Journal of Macromolecular Science, Part C: Polymer Reviews*, 44, 351-388.
- URBANEK, A. K., RYMOWICZ, W. & MIROŃCZUK, A. M. 2018. Degradation of plastics and plastic-degrading bacteria in cold marine habitats. *Applied Microbiology and Biotechnology*, 102, 7669-7678.



- URBANEK, A. K., RYMOWICZ, W., STRZELECKI, M. C., KOCIUBA, W., FRAN CZAK, Ł. & MIROŃCZUK, A. M. 2017. Isolation and characterization of Arctic microorganisms decomposing bioplastics. *AMB Express*, 7, 148.
- US-EPA 1992. Plastic pellets in the aquatic environment: sources and recommendations. *United States Environmental Protect Agency Office of Water*, <http://www.globalgarbage.org/13%20EPA%20Plastic%20Pellets.pdf>.
- VAGUE, M., CHAN, G., ROBERTS, C., SWARTZ, N. A. & MELLIES, J. L. 2019. *Pseudomonas* isolates degrade and form biofilms on polyethylene terephthalate (PET) plastic. *bioRxiv*, 647321.
- VAN CAUWENBERGHE, L., VANREUSEL, A., MEES, J. & JANSSEN, C. R. 2013. Microplastic pollution in deep-sea sediments. *Environmental Pollution*, 182, 495-499.
- VAN FRANEKER, J. A., BLAIZE, C., DANIELSEN, J., FAIRCLOUGH, K., GOLLAN, J., GUSE, N., HANSEN, P.-L., HEUBECK, M., JENSEN, J.-K. & LE GUILLOU, G. 2011. Monitoring plastic ingestion by the northern fulmar *Fulmarus glacialis* in the North Sea. *Environmental Pollution*, 159, 2609-2615.
- VAN SEBILLE, E., WILCOX, C., LEBRETON, L., MAXIMENKO, N., HARDESTY, B. D., VAN FRANEKER, J. A., ERIKSEN, M., SIEGEL, D., GALGANI, F. & LAW, K. L. 2015. A global inventory of small floating plastic debris. *Environmental Research Letters*, 10, 124006.
- VANDECANDELAERE, I., NERCESSIAN, O., SEGAERT, E., ACHOUAK, W., FAIMALI, M. & VANDAMME, P. 2008. *Ruegeria scottomollicae* sp. nov., isolated from a marine electroactive biofilm. *International Journal of Systematic and Evolutionary Microbiology*, 58, 2726-2733.
- VEITH, B., HERZBERG, C., STECKEL, S., FEESCHE, J., MAURER, K. H., EHRENREICH, P., BÄUMER, S., HENNE, A., LIESEGANG, H., MERKL, R., EHRENREICH, A. & GOTTSCHALK, G. 2004. The complete genome sequence of *Bacillus licheniformis* DSM13, an organism with great industrial potential. *Journal of Molecular Microbiology and Biotechnology*, 7, 204-211.
- VÉLEZ-RUBIO, G. M., ESTRADES, A., FALLABRINO, A. & TOMÁS, J. 2013. Marine turtle threats in Uruguayan waters: insights from 12 years of stranding data. *Marine Biology*, 160, 2797-2811.
- VENKATACHALAM, S., NAYAK, S. G., LABDE, J. V., GHARAL, P. R., RAO, K. & KELKAR, A. K. 2012. Degradation and recyclability of poly (ethylene terephthalate). *Polyester*. IntechOpen, Rijeka, Croatia, pp 107–130.
- VILLETTI, M. A., CRESPO, J. S., SOLDI, M. S., PIRES, A. T. N., BORSALI, R. & SOLDI, V. 2002. Thermal Degradation of Natural Polymers. *Journal of Thermal Analysis and Calorimetry*, 67, 295-303.
- VOLOVA, T. G., BOYANDIN, A. N., VASILIEV, A. D., KARPOV, V. A., PRUDNIKOVA, S. V., MISHUKOVA, O. V., BOYARSKIKH, U. A., FILIPENKO, M. L., RUDNEV, V. P., BÁ XUÂN, B., VIỆT DŨNG, V. & GITELSON, I. I. 2010. Biodegradation of polyhydroxyalkanoates (PHAs) in tropical coastal waters and

- identification of PHA-degrading bacteria. *Polymer Degradation and Stability*, 95, 2350-2359.
- WALSH, E. A., KIRKPATRICK, J. B., RUTHERFORD, S. D., SMITH, D. C., SOGIN, M. & D'HONDT, S. 2015. Bacterial diversity and community composition from seafloor to subseafloor. *The ISME Journal*, 10, 979.
- WANG, Q., GARRITY, G. M., TIEDJE, J. M. & COLE, J. R. 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Applied and Environmental Microbiology*, 73, 5261-5267.
- WANG, Z., XU, J., LI, Y., WANG, K., WANG, Y., HONG, Q., LI, W.-J. & LI, S.-P. 2010. *Rhodococcus jialingiae* sp. nov., an actinobacterium isolated from sludge of a carbendazim wastewater treatment facility. *International Journal of Systematic and Evolutionary Microbiology*, 60, 378-381.
- WATANABE, K. 2001. Microorganisms relevant to bioremediation. *Current Opinion in Biotechnology*, 12, 237-241.
- WATERS, C. M. & BASSLER, B. L. 2005. QUORUM SENSING: Cell-to-Cell Communication in Bacteria. *Annual Review of Cell and Developmental Biology*, 21, 319-346.
- WAY, C., WU, D. Y., DEAN, K. & PALOMBO, E. 2010. Design considerations for high-temperature respirometric biodegradation of polymers in compost. *Polymer Testing*, 29, 147-157.
- WEBB, H., ARNOTT, J., CRAWFORD, R. & IVANOVA, E. 2013. Plastic Degradation and Its Environmental Implications with Special Reference to Poly(ethylene terephthalate). *Polymers*, 5, 1.
- WEBB, H. K., CRAWFORD, R. J., SAWABE, T. & IVANOVA, E. P. 2009. Poly(ethylene terephthalate) polymer surfaces as a substrate for bacterial attachment and biofilm formation. *Microbes Environ*, 24, 39.
- WEI, R. & ZIMMERMANN, W. 2017. Microbial enzymes for the recycling of recalcitrant petroleum-based plastics: how far are we? *Microbial Biotechnology*, 10, 1308-1322.
- WEINBAUER, M. G., HORNÁK, K., JEZBERA, J., NEDOMA, J., DOLAN, J. R. & ŠIMEK, K. 2007. Synergistic and antagonistic effects of viral lysis and protistan grazing on bacterial biomass, production and diversity. *Environmental Microbiology*, 9, 777-788.
- WEINSTEIN, J. E., CROCKER, B. K. & GRAY, A. D. 2016. From macroplastic to microplastic: Degradation of high-density polyethylene, polypropylene, and polystyrene in a salt marsh habitat. *Environmental Toxicology and Chemistry*, 35, 1632-1640.
- WELDEN, N. A. & COWIE, P. R. 2017. Degradation of common polymer ropes in a sublittoral marine environment. *Marine Pollution Bulletin*, 118, 248-253.
- WHITMAN, W. B., COLEMAN, D. C. & WIEBE, W. J. 1998. Prokaryotes: the unseen majority. *Proceedings of the National Academy of Sciences*, 95, 6578-6583.

- WIEDENBECK, J. & COHAN, F. M. 2011. Origins of bacterial diversity through horizontal genetic transfer and adaptation to new ecological niches. *FEMS microbiology reviews*, 35, 957-976.
- WILCOX, C., HEATHCOTE, G., GOLDBERG, J., GUNN, R., PEEL, D. & HARDESTY, B. D. 2015a. Understanding the sources and effects of abandoned, lost, and discarded fishing gear on marine turtles in northern Australia. *Conservation Biology*, 29, 198-206.
- WILCOX, C., VAN SEBILLE, E. & HARDESTY, B. D. 2015b. Threat of plastic pollution to seabirds is global, pervasive, and increasing. *Proceedings of the National Academy of Sciences*, 112, 11899.
- WILKES, R. A. & ARISTILDE, L. 2017. Degradation and metabolism of synthetic plastics and associated products by *Pseudomonas* sp.: capabilities and challenges. *Journal of applied microbiology*, 123, 582-593.
- WILLIAMS, D. F. & ZHONG, S. P. 1994. Biodeterioration/biodegradation of polymeric medical devices in situ. *International Biodeterioration & Biodegradation*, 34, 95-130.
- WITT, V., WILD, C., ANTHONY, K. R., DIAZ-PULIDO, G. & UTHICKE, S. 2011. Effects of ocean acidification on microbial community composition of, and oxygen fluxes through, biofilms from the Great Barrier Reef. *Environmental Microbiology*, 13, 2976-2989.
- WOODALL, L. C., SANCHEZ-VIDAL, A., CANALS, M., PATERSON, G. L., COPPOCK, R., SLEIGHT, V., CALAFAT, A., ROGERS, A. D., NARAYANASWAMY, B. E. & THOMPSON, R. C. 2014. The deep sea is a major sink for microplastic debris. *Royal Society open science*, 1, 140317.
- WORM, B., LOTZE, H. K., JUBINVILLE, I., WILCOX, C. & JAMBECK, J. 2017. Plastic as a Persistent Marine Pollutant. *Annual Review of Environment and Resources*, 42, 1-26.
- WRIGHT, S. L., ROWE, D., THOMPSON, R. C. & GALLOWAY, T. S. 2013a. Microplastic ingestion decreases energy reserves in marine worms. *Current Biology*, 23, R1031-R1033.
- WRIGHT, S. L., THOMPSON, R. C. & GALLOWAY, T. S. 2013b. The physical impacts of microplastics on marine organisms: A review. *Environmental Pollution*, 178, 483-492.
- WU, P., HUANG, J., ZHENG, Y., YANG, Y., ZHANG, Y., HE, F., CHEN, H., QUAN, G., YAN, J., LI, T. & GAO, B. 2019. Environmental occurrences, fate, and impacts of microplastics. *Ecotoxicology and Environmental Safety*, 184, 109612.
- WYPYCH, G. 2013. 2 - PHOTOCHEMISTRY. *Handbook of Material Weathering (Fifth Edition)*. Oxford: Elsevier.
- XIA, X.-L., LIU, W.-T., TANG, X.-Y., SHI, X.-Y., WANG, L.-N., HE, S.-Q. & ZHU, C.-S. 2014. Degradation behaviors, thermostability and mechanical properties of poly (ethylene terephthalate)/polylactic acid blends. *Journal of Central South University*, 21, 1725-1732.

- XIE, H., ZAFIRIOU, O. C., CAI, W.-J., ZEPP, R. G. & WANG, Y. 2004. Photooxidation and Its Effects on the Carboxyl Content of Dissolved Organic Matter in Two Coastal Rivers in the Southeastern United States. *Environmental Science & Technology*, 38, 4113-4119.
- YANG, J., YANG, Y., WU, W.-M., ZHAO, J. & JIANG, L. 2014. Evidence of polyethylene biodegradation by bacterial strains from the guts of plastic-eating waxworms. *Environmental science & technology*, 48, 13776-13784.
- YANG, T., REN, L., JIA, Y., FAN, S., WANG, J., WANG, J., NAHURIRA, R., WANG, H. & YAN, Y. 2018. Biodegradation of Di-(2-ethylhexyl) Phthalate by *Rhodococcus ruber* YC-YT1 in Contaminated Water and Soil. *International journal of environmental research and public health*, 15, 964.
- YANG, Y., YANG, J., WU, W.-M., ZHAO, J., SONG, Y., GAO, L., YANG, R. & JIANG, L. 2015. Biodegradation and Mineralization of Polystyrene by Plastic-Eating Mealworms: Part 2. Role of Gut Microorganisms. *Environmental Science & Technology*, 49, 12087-12093.
- YANO, K., WACHI, M., TSUCHIDA, S., KITAZUME, T. & IWAI, N. 2015. Degradation of benzonitrile via the dioxygenase pathway in *Rhodococcus* sp. 065240. *Bioscience, Biotechnology, and Biochemistry*, 79, 496-504.
- YE, S. & ANDRADY, A. L. 1991. Fouling of floating plastic debris under Biscayne Bay exposure conditions. *Marine Pollution Bulletin*, 22, 608-613.
- YOSHIDA, S., HIRAGA, K., TAKEHANA, T., TANIGUCHI, I., YAMAJI, H., MAEDA, Y., TOYOHARA, K., MIYAMOTO, K., KIMURA, Y. & ODA, K. 2016. A bacterium that degrades and assimilates poly(ethylene terephthalate). *Science*, 351, 1196-1199.
- ZBYSZEWSKI, M. & CORCORAN, P. L. 2011. Distribution and Degradation of Fresh Water Plastic Particles Along the Beaches of Lake Huron, Canada. *Water, Air, & Soil Pollution*, 220, 365-372.
- ZETTLER, E. R., MINCER, T. J. & AMARAL-ZETTLER, L. A. 2013. Life in the "Plastisphere": Microbial Communities on Plastic Marine Debris. *Environmental Science & Technology*, 47, 7137-7146.
- ZHOU, Q., ZHANG, H., FU, C., ZHOU, Y., DAI, Z., LI, Y., TU, C. & LUO, Y. 2018. The distribution and morphology of microplastics in coastal soils adjacent to the Bohai Sea and the Yellow Sea. *Geoderma*, 322, 201-208.
- ZHOU, S., SONG, J., DONG, W., MU, Y., ZHANG, Q., FAN, Z., WANG, Y., KONG, D., ZHOU, Y., JIANG, X., ZHAO, B., HAN, G. & RUAN, Z. 2017. Nicosulfuron Biodegradation by a Novel Cold-Adapted Strain *Oceanisphaera psychrotolerans* LAM-WHM-ZC. *Journal of Agricultural and Food Chemistry*, 65, 10243-10249.